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(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: JACOBS, Susan, Catherine [GB/GB]; CBD, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: BOWDERY, A., O.; D/IPR, Formalities Section (DERA), Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

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(57) Abstract

A nucleic acid which encodes a polypeptide which produces a protective immune response against an alpha-virus such as a Venezuelan Equine Encephalitis Virus, in a mammal to which it is administered, said nucleic acid lacking a competant nuclear targeting signal in the capsid gene. The nucleic acids of the invention are expressed at enhanced levels in vectors such as adenovirus vectors. In particular there is described a deletion mutant of an alpha-virus. Vectors such as recombinant adenoviruses which express the deletion mutant are potentially of use as vaccines.

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Recombinant Virus

The present invention relates to the production of vectors which express alpha-virus genes, such as recombinant viral vectors like adenovirus. The invention further relates to prophylactic and therapeutic vaccines which are protective against these alpha-viruses, such Venezuelan Equine Encephalitis Virus (VEEV), as well as nucleic acids which are used in the vectors, and methods of treatment using the vaccines.

The structural proteins of the alphaviruses are translated from a 26s RNA. The genes encoding these proteins are contained within a single open reading frame in the order:

capsid-E3-E2-6K-E1.

The capsid protein is also known as the "core" and both terms are used herein.

Each protein is either co- or post-translationally cleaved from the poly-protein precursor.

Many prophylactic and therapeutic vaccines rely on the use of recombinant viruses such as vaccinia virus, or adenovirus including replication competant and replication defective adenovirus, for effective delivery of the immunogens.

However, there are sometimes difficulties associated with the expression of alpha viruses in such vectors.

The applicants have found that deletion of a region of an alphavirus gene improves expression in certain vectors and allows expression in other vectors which could otherwise not be made. This is useful in vaccine production.

Thus according to the present invention there is provided a nucleic acid which encodes a polypeptide which produces a

protective immune response against an alpha-virus in a mammal to which it is administered, said nucleic acid lacking a competant nuclear targeting signal from a capsid gene thereof.

The nucleic acid as described above may be expressed at enhanced levels, for example in an adenovirus.

As used herein, the term "polypeptide" encompasses short polypeptides as well as proteins. The expression "enhanced" means that the expression level of the polypeptide is increased as compared to that which would occur if an otherwise similar nucleic acid including the said portion of the nuclear targeting signal were present.

The nuclear targeting signal (NTS) of any particular alphavirus is either known or it can be determined by alignment with sequences which resemble known nuclear targeting signals in other alphaviruses. For instance, Jakob, Preparative Biochemistry (1995) 25: 99-117 shows the nuclear targeting signal in the Semliki forest virus. An example of a nuclear targeting sequence of VEEV is described below. These sequences are present in the gene encoding the core or capsid protein.

In general, the nuclear targeting signal will be located in a lysine rich area of the genome and will comprise a region which has at least 3 and generally 4 adjacent lysines such as described by Chelsky et al., 1989, Mol + Cell Biology, 9, p2487-2492. In the nucleic acids of the invention, the NTS is inactivated either by complete or partial deletion, or by mutation, for example to alter at least some of the lysine residues.

The nucleic acid of the invention may be a DNA or an RNA molecule, suitably a cDNA. Furthermore it suitably encodes a polypeptide which comprises at least one structural protein of said alpha-virus, and most preferably all of

these. Thus, in a preferred embodiment, the recombinant nucleic acid of the invention encodes at least the capsid-E3-E2 proteings of the alpha-virus and more preferably the capsid-E3-E2-6K-E1 proteins of an alpha-virus, provided that the region which encodes the capsid protein lacks a competant nuclear targeting domain.

One alphavirus which has been found to benefit particularly from the present invention is a Venezulan Equine Encephalitis Virus (VEEV). This virus, is a mosquito-borne alphavirus which is an important cause of epidemic disease in humans and of epizootics in horses, donkeys and mules in certain parts of the world, in particular the South Americas.

The existing VEE vaccine, TC-83, was initially produced by attenuation of the Trinidad donkey strain (TRD) of VEE by sequential passage in guinea pig heart cell cultures. However, this vaccine is generally regarded as being inadequate for human vaccination. This is mainly due to the high incidence of side effects in vaccinees and the large proportion of vaccinees who fail to develop neutralising antibodies (Monath et al. 1992, Vaccine Research, 1, 55-68).

A vaccinia-based vaccine against VEE has been constructed (Kinney et al. J. Gen. Virol. 1988, 69, 3005-3013). In this recombinant, 26S RNA encoding structural genes of VEE were inserted into the NYCBH strain of vaccinia. The recombinant virus protected against sub-cutaneous challenge but had limited efficacy against aerosol challenge with VEE.

Attempts to express the full length sequence of this virus in adenovirus, a particularly useful virus for vaccine production, failed completely. However, certain deletion mutants could be successfully expressed. It has been found that the virus proteins will be expressed from a recombinant adenovirus which lacks at least some, and suitably all of nucleotides 7749-7887 within the VEEV genome.

All DNA sequence co-ordinants on the VEEV TC-83 strain followed those of the Trinidad Donkey virus strain (R.M. Kinney et al. Virology (1989) **170**, 19-30). The cDNA of the 26s mRNA encoding the TC-83 structural region is that reported by Kinney et al (1988) supra, the content of which is incorporated herein by reference. The virulent Trinidad donkey strain of VEE and the attenuated strain TC-83 have both been cloned and sequenced and the amino acid and nucleotide numbering system used in this reference will be used hereinafter. This work revealed that there are seven amino acid changes between TRD and TC-83. The majority (five) of these changes occur within the gene encoding the glycoprotein E2. The applicants have found a further three changes over and above those described by Kinney as detailed below.

The deletion or omission of a nuclear targeting domain has also been found to improve the expression of other VEEV encoded proteins in other vectors such as plasmids.

Deletion of corresponding regions in other alphaviruses should produce similar enhancements in expression.

The recombinant nucleic acids of the invention may be prepared by any of the well known techniques used in recombinant DNA technology. They may be prepared ab initio using the available chemical methods, for example the automated chemical synthesisers. Alternatively, they may be prepared from wild-type alpha viruses, using known recombinant DNA techniques to generate deletion mutants.

Thus in a further aspect the invention provides a deletion mutant of an alpha-virus, which mutant lacks a nuclear targeting domain, such as a region corresponding to nucleotides 7749-7887 of VEEV. Corresponding regions in other alphaviruses could be readily be determined by comparing sequences and determining analogous regions as is understood in the art. Such comparisons can be made by computer programs.

Confirmation of the nature of the nuclear targeting domain can be confirmed, for example using labelled fragments which may or may not include the purported nuclear targeting domain, and examining where the label appears when the fragments are located in cells transfected with the fragments. The labels may be radiolabels or fluorescent labels as are well known in the art.

Preferably the deletion mutant of the invention is a deletion mutant of VEEV.

A further aspect of the invention provides a recombinant nucleic acid which encodes a deletion mutant of an alpha-virus as described above.

Nucleic acids of the invention are suitably incorporated into vectors, in particular virus vectors like adenovirus (which may be either replication competant or replication defective), vaccinia virus, or in other expression plasmids where they are under the control of a suitable promoter as understood in the art. Preferably the nucleic acids of the invention is incorporated into an adenovirus and most preferably a replication defective adenovirus.

These viruses or plasmids can form vaccines. For this purpose, they will suitably be combined with a pharmaceutically acceptable carrier. Virus vectors are preferably combined with a liquid carrier in an injectable formulation. Plasmid vectors may also be made into an injectable formulation or they may alternatively be bound onto a solid carrier such as a gold bead, which are suitable for administration by means of a gene gun, to the skin of a patient.

In yet a further aspect, the invention provides a method of producing a protective immune response to an alpha-virus, said method comprising administering to a mammal a vaccine

as described above. The protective immune response may be used both in prophylaxis and in therapy. Suitable doses will be determined by clinicians taking into account the nature of the patient, the nature of the alphavirus and in the case of therapeutic treatment, as well as the precise nature and form of the vaccine. However in general, when using a virus vector, dosages of the vector will be of at least 10^4 pfu. For instance, in the case of vaccinia vectors or replication competant adenovirus, dosages are suitably in the range of from 10^4-10^{12}pfu (pfu = particle forming units). Replication defective adenovirus may have to be administered at higher dosages.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 is a diagrammatic map (not to scale and not inclusive) showing the structure of VEEV cDNA including restriction sites and the coding regions for the various proteins;

Figure 2 shows the sequences of various linkers and adaptors used in the constructs of the examples; and

Figures 3-28 are the cloning diagrams referred to in the Examples.

In the following Examples, the abbreviations used are as follows:

VEEV	Venezuelan Equine Encephalitis Virus
TRD	Trinidad Donkey Virus Strain
C	Core
E1	Structural protein 1
E2	Structural protein 2
E3	Structural protein 3
6K	Structural protein 6K
CMV	Cytomegalovirus

IEP	Immediate Early Promoter
Term	Terminator signal
cDNA	Complementary DNA
RAd	Recombinant E1a- Adenovirus
NCR	Non-Coding Region
ATG	Start Codon
MCS	Multiple Cloning Site
NTS	Nuclear Targeting Signal
RBS	Ribosome Binding Site
nt	Nucleotide
nts	Nucleotides
PCR	Polymerase Chain Reaction
Δ	Minus

The cDNA of the 26s sub-genomic RNA encoding the TC-83 structural region is that used in the work described below and was provided on the plasmid pTC-5a as reported by Kinney et al. (1988). J.G.V. 69: 3005-3114. All VEEV TC-83 cDNA sequence co-ordinates described below followed those Kinney and co-workers (Kinney et al. (1989). Virology 170: 19-33) for the VEEV strain Trinidad donkey (TRD). Nucleotide sequence analysis of the TC-83 VEEV cDNA identified three differences in addition to those described (Kinney et al. (1989). Virology 170: 19-33) within the open reading frames of TC-83 and TRD. These changes are; i) within the 6K gene of TC-83 an addition codon GCG is located between nucleotides (nts) 9989 and 9990, ii)within the E1 gene of TC-83 nt 10353 is T, and ii) in E1 gene of TC-83 nt 18897 is G.

Example 1

Preparation of deletion mutants

All constructs were cloned into the expression cassette contained within pMV100 or pEVV101. pMV100 has been described previously (Wilkinson and Akrigg. (1992) N.A.R. 20: 2233-2239, Jacobs et al. (1992) J. Virol 66: 2086-2095). The insertion at the XbaI site in pMV100 of the palindromic adapter L1 (fig 2a) which encodes an EcoRI site on an XbaI

fragment generated pEVV101 (Figure 3). Thus this modification adds an EcoRI site in between the CMV immediate early promoter (IEP) and terminator signal (Term) allowing cDNA to be cloned into the expression cassette (or to be excised from it) on a BamHI, XbaI or EcoRI restriction fragment.

Recombinant E1a- adenovirus 5 (Rad) was generated using materials, including the shuttle vector pMV60, and methods described elsewhere(Wilkinson and Akrigg. (1992) N.A.R. 20: 2233-2239, Jacobs et al. (1992) J. Virol 66: 2086-2095). The VEEV cDNA from pTC-5a was cloned into pEVV101 on an EcoRI fragment generating pEVV102 (Figure 4). The expression cassette containing the VEEV cDNA was subcloned from pEVV102 into pMV60 on a HindIII fragment generating pEVV105. RAd could not be generated with pEVV105 or other plasmids similarly constructed. RAd was made, however, when the VEEV cDNA was cloned in the wrong orientation under the control of the promoter.

The truncated 5′ non-coding region (NCR) and core gene (5′-C) were deleted from the TC-83 VEEV cDNA to investigate whether RAd could be made containing the E3-E2-6K-E1 and the truncated 3′ NCR (E3-E2-6K-E1-3′). pEVV105 was digested with SpeI cutting the VEEV cDNA immediately downstream of nt 8389, three nucleotides into the 5′ of the E3 gene. The three deleted nucleotides were restored by the insertion of the palindromic adapter L4A (fig 2b) at the SpeI site generating pEVV110 (Figure 5). L4A also encoded a start codon immediately upstream of the three 5′ nts of the E3 gene, which is preceded by a Kozak consensus sequence ACC (Kozak. (1984). Nature 308: 241-246, Kozak. (1986). Cell 44: 283-292, Kozak. (1987). J. Mol. Biol. 196: 947-950) and an EcoRI site. Using the EcoRI site in L4A and that at the 3′ of the VEEV cDNA, the cDNA VEEV construct E3-E2-6K-E1-3′ was removed from pEVV110 and cloned into pEVV101 generating pEVV108 (Figure 6). The expression cassette containing the VEEV cDNA was subcloned from pEVV108 to pMV60 on a HindIII

fragment generating pEVV109. RAd was generated using pEVV109.

An attempt was made to generate RAd expressing the truncated 5'NCR and core (5'-C) . To clone 5'-C, the plasmid pUC19 (provided by Boehringer Mannheim UK) was modified by reversing the order of the XbaI and AccI sites within the multiple cloning site (MCS). A BamHI and SphI adapter encoding an AccI and XbaI site (L7L8, fig 2c) was inserted into the MCS of pUC19 to generate pEVV118 (Figure 7). The adapter L5L6 (fig 2d) provided the 3' 26 nucleotides of core gene from the AccI site (nt 8361) to the 3' of core at nt 8386 and an appropriate stop codon on an AccI/XbaI fragment and was cloned into pEVV118 generating pEVV119 (Figure 8). The 5' NCR and remaining 5' of the core gene, up to and including nt8360, was removed from pEVV102 on a BamHI-AccI fragment and cloned into pEVV119 completing the constructing of 5'-C and generating pEVV120 (Figure 9). The 5'-C fragment was removed from pEVV120 on an XbaI fragment and cloned into pMV100 generating pEVV121 (Figure 10). The expression cassette containing 5'-C was subcloned from pEVV121 into pMV60 on a HindIII fragment generating pEVV123. RAd could not be generated using pEVV123.

In an attempt to identify the domain responsible for the failure to generate RAd containing core, the VEEV core gene was cloned in two halves. The adapter L10L13 (fig 2e) was inserted at the NsiI site in core between nucleotides 8016 and 8017. This placed an in frame stop codon downstream of the NsiI site followed by an XbaI site, an in frame start codon and a second NsiI site. The resultant plasmid (pEVV129, Figure 11) was digested with XbaI generating two fragments containing C sequence. One containing the 5' NCR and the 5' of core up to and including nt 8016 which immediately precedes an inframe stop codon. The second containing the 3' of the core gene from nt 8017 which is immediately preceded by an in frame start codon, up to and including the stop codon inserted in the construction of

5'-C (i e. pEVV121). Each fragment was cloned into pMV100 on an XbaI fragment generating pEVV125 (Figure 12) which encodes the 5' of core and pEVV126 (Figure 12) which encodes the 3' of core. The expression cassette were subcloned from pEVV125 and pEVV126 into pMV60 on a HindIII fragment generating pEVV127 and pEVV128 respectively. RAd could not be generated using pEVV127 but was generated from the pEVV128.

Three domains within the 5' of core encoded by pEVV125 that might be responsible for the failure to generated RAd virus containing core were identified. These domains are i) the 17 nts upstream of the start codon at nt7562 which form the truncated 5' NCR; ii) the putative ribosome binding site (RBS) between nt 7886-7930 identified by sequence alignment with the alphaviruse Sindbis (Wengler et al. (1992). Virol ; 191: 880-888, Geigenmuller-Gnirke et al. (1993). J Virol 67: 1620-1626, Owen and Kuhn. (1996). J. Virol 70: 2757-2763); iii) a putative nuclear targeting signal (NTS) between nt 7751-7885 identified by sequence alignment with the alphavirus Semliki forest virus (Jakob. (1995). Preparative Biochemistry 25: 99-117).

To remove the 5' NCR from core, a PCR fragment was generated with primer P2 (fig 2h) and P3 (fig 2i) using pEVV123 as the template. From 5' to 3', P2 contains a GC rich tail, BamHI site, and the 17 nts homologous to the positive strand of the 5' of the core gene from the start codon at nt 7562. From 5' to 3', P3 contains a GC rich tail and BamHI site followed by 14 nucleotides complementary the positive strand the core gene between nt 7726-7739 including a Bsu 36I site located between nt 7733-7739. The resultant PCR product therefore contained BamHI sites which flanked sequence containing the 5' of core from the start codon at nt 7562 up to and including the internal Bsu 36I site (nt 7739). This PCR product was digested with BamHI and cloned into pUC18 (provided by Boehringer Mannheim UK). The BamHI fragment was then subcloned into pUC19 generating pEVV135 (Figure

13). Reconstruction of core gene was completed by inserting the remaining 3′ of core from the Bsu 36I site (nt 7735) up to and including the stop codon following nt8386 from pEVV121 on a Bsu 36I - HindIII fragment into pEVV135 generating pEVV136 (Figure 14). The core gene was removed on a BamHI fragment from pEVV136 and cloned into pMV100 generating pEVV133 (Figure 15). The expression cassette containing the Core was subcloned from pEVV133 into pMV60 on a HindIII fragment generating pEVV134. RAd could not be generated using pEVV134

To delete the RBS from core, a TfiI site within core was used which necessitated the removal of the TfiI sites at nt641 and 781 in pUC19. pUC19 was digested with TfiI and the sticky ends generated were modified with Klenow before the plasmid was re-ligated generating pEVV145. Core was cloned into the MCS of pEVV145 on an XbaI fragment from pEVV121 generating pEVV146 (Figure 16). To delete either the NTS or RBS from core it was necessary to remove both signals on a Bsu 36I -TfiI fragment (nts 7735-7928) and then restore either the NTS or RBS separately. This was achieved by digesting pEVV146 with Bsu 36I and TfiI and replacing the RBS-NTS fragment with either a Bsu 36I-TfiI PCR fragment encoding the NTS or a Bsu 36I-TfiI adapter encoding the RBS.

A Bsu 36I-TfiI PCR fragment containing the NTS was generated using primers L20 and L21 (fig 1j and 1k). L20 is homologous to the positive strand of core gene and encodes a Bsu 36I site followed by the 10 nt from 7735 to 7749, L21 encoded 25nts complementary to the positive strand of the core gene between nts7872-7897 followed by a TfiI site . The PCR fragment was cloned into pTAg (R & D Systems Europe LTD) using the nontemplated-dependent single A added to the 3′ ends of the PCR product. The Bsu 36I-TfiI fragment containing the NTS was then excised from pTAg and cloned into pEVV146 replacing the Bsu 36I-TfiI fragment containing the NTS and RBS and generating core minus (Δ RBS

(CΔRBS)(pEVV148 (Figure 17)). CΔRBS was removed from pEVV148 and cloned into pMV100 on XbaI fragment generating pEVV149 (Figure 18). The expression cassette containing Core Δ RBS was subcloned from pEVV149 into pMV60 on a HindIII fragment generating pEVV150. RAd could not be generated using pEVV150.

Core ΔNTS was generated by cloning the adapter L28L29 (fig 2f) into Bsu 36I-TfiI digested pEVV146 generating pEVV151 (Figure 19). L28L29 contains nts7735 to 7750 which are adjacent to and continuous with the 42 from nt 7886 to 7921 The NTS is located between nt 7751 and 7885 therefore L28L29 encodes the region of the core gene between the Bsu 36I site and the NTS and the NTS and the TfiI site thus containing no sequence from the NTS and generating CΔ□NTS . CΔNTS was cloned into pMV100 from pEVV151 on an XbaI fragment generating pEVV152 (Figure 20). The expression cassette containing the CΔNTS was subcloned from pEVV152 into pMV60 on a HindIII fragment generating pEVV153. RAd was generated using pEVV153.

To confirm that the NTS encoded by core was responsible for the failure to generate recombinant adenovirus containing the VEEV core, CΔNTS was restored to the construct E3-E2-6K-E1-3′ using adapter L30L31 (fig 2g). Adapter L30L31 contains the 34nt downstream of the AccI site at nt8361 in core to nt 8394 of the SpeI site located in E1 and therefore re-joins core to E3. CDNTS was removed from pEVV152 on an XbaI fragment replacing 3′-C in pEVV120 generating pEVV159 (Figure 21). L30L31 on a AccI-HindIII fragment was cloned into pEVV159 between the AccI site at the 3′ of core and the HindIII site within the MCS of pEVV120 generating pEVV160 (Figure 22). CDNTS +L30L31 was removed from pEVV160 on XbaI-SpeI fragment and cloned into pEVV108 placing core-L30L31 upstream of E3 and restoring the Core-E3 junction generating pEVV161 (Figure 23). The VEEV cDNA CΔNTS-E3-E2-

6K-E1-3′ was removed from pEVV160 on an EcoRI fragment and cloned into pEVV101 generating pEVV163 (Figure 24). The expression cassette containing CΔNTS-E3-E2-6K-E1-3′ was subcloned from pEVV163 into pMV60 on a HindIII fragment generating pEVV162. RAd was generated using pEVV162.

Example 2

Confirmation of Nuclear targeting domain

To determine the potential of the putative NTS to locate the Core protein to the cell nucleus, core was expressed as a fusion protein with Green Fluorescent Protein (GFP). The start codon (ATG) was removed from core by replacing the 5′ of core in pEVV120 from the BamHI site immediately upstream of the start codon to the unique Bsu361 site within the core gene with a PCR fragment containing an identical fragment of core with the exception that the start codon had been removed. This PCR fragment was generated with the Primers P3 and P15. Within the primer P15 immediately upstream of the BamHI site and downstream of the first nucleotide of Core and additional two nucleotides were included (AT) in order to shift the reading frame two places to the right. This cloning generated the plasmid pEVV188 (Figure 25).

Core minus it's start codon was removed from pEVV188 on a BamHI - XbaI fragment and cloned into pUC19 generating pEVV187 (Figure 26). This cloning placed a SalI site downstream of the XbaI site allowing core minus its start codon to be removed from pEVV187 on a BamHI-SalI fragment and cloned into the commercially available vector pEGFP-C2 (Clontech, Palo Alto CA. USA) generating pEVV186 (Figure 27). This cloning placed core minus its start codon downstream of and in frame with the GFP gene so that core is expressed as a C-terminal fusion with GFP.

When pEVV186 transfected cells were examined by immunofluorescence GFP was found to locate to the nucleus of the cell.

That targeting of GFP-core fusion protein to the nucleus was directed by the NTS encoded by core was demonstrated by generating pEVV172. pEVV172 (Figure 28) was generated by cloning the NTS removed from pTAg (Figure 17) on an EcoRI and SalI fragment and cloned into pEGFP-C2. The NTS was therefore expressed as a C-terminal fusion with GFP. A control plasmid pEVV173 was generated by removing the NTS on a BamHI and BglII fragment and cloning it back into pEGFP-C2 in the antisence orientation behind GFP. In cells transfected with pEVV173, GFP was located throughout the cell and particularly in the cytoplasm. In cells transfected with pEVV172, GFP was located to the nucleus.

Example 3

Immunisation studies

Groups of 10 balb/c mice were immunised with plasmids using the Helios Gene Gun (Bio-Rad, Hercules, CA. USA). The plasmid used were pEVV100, pEVV102 and pEVV163. Each mouse received approximately 5μg plasmid at day 0 and again after 11 weeks. Mice were then challenged 22 weeks following the original immunisation, with $30LD_{50}$ of the virulent stain of VEEV TRD subcutaneously. The results are tabulated in table 1. This data suggests that the immune response elicited following immunisation with a plasmid pEVV163 elicited protection while pEVV102 did not. The protective effect seen appears not to be solely the result of an antibody response.

Table 1.

Plasmid	Number of mice surviving 20 days post challenge / Total number of mice
pEVV100	0/10
pEVV102	0/10
pEVV163	3/10

Claims

1. A nucleic acid which encodes a polypeptide which produces a protective immune response against an alpha-virus in a mammal to which it is administered, said nucleic acid lacking a competant nuclear targeting signal in the capsid gene.

2. A nucleic acid according to claim 1 which can be expressed in an adenovirus vector.

3. A nucleic acid according to claim 1 or claim 2 which encodes at least the capsid-E3-E2 proteins of an alphavirus, provided that the region which encodes the capsid protein lacks a nuclear targeting signal.

4. A nucleic acid according to claim 3 which encodes the capsid-E3-E2-6K-E1 proteins of an alpha-virus, provided that the region which encodes the capsid protein lacks a nuclear targeting signal.

5. A nucleic acid according to any one of the preceding claims wherein the alpha-virus is a Venezulan Equine Encephalitis Virus (VEEV).

6. A deletion mutant of an alpha-virus, which mutant lacks a nuclear targeting signal from the capsid gene.

7. A deletion mutant of an alpha-virus which lacks a region corresponding to nucleotides 7749-7887 of VEEV.

8. A deletion mutant according to claim 7 wherein the alpha-virus is VEEV.

9. A nucleic acid which encodes a deletion mutant of an alpha-virus as claimed in any one of claims 6 to 8.

10. A recombinant adenovirus which comprises a nucleic acid according to any one of claims 1 to 5 or 9.

11. A recombinant vaccinia virus which comprises a nucleic acid according to any one or claims 1 to 5 or 9.

12. A plasmid which comprises a nucleic acid according to any one or claims 1 to 5 or 9 under the control of a promoter which allows expression thereof.

13. A vaccine which comprises a virus according to claim 10 or claim 11 or a plasmid according to claim 12.

14. A vaccine according to claim 13 which further comprises pharmaceutically acceptable carrier.

15. A method of producing a protective immune response to an alpha-virus, said method comprising administering to a mammal a vaccine according to claim 13 or claim 14.

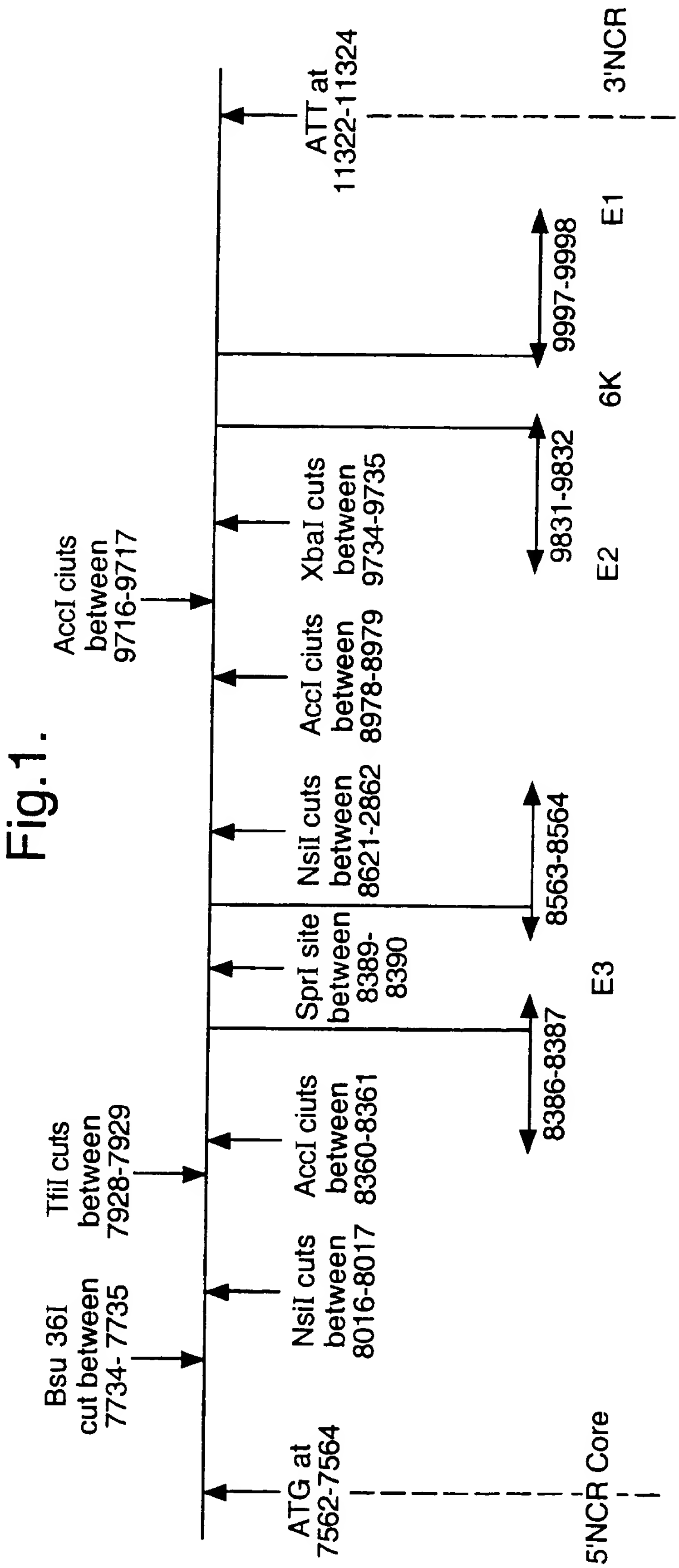

ATG at
7562-7564
5'NCR Core
Bsu 36I
cut between
7734- 7735
NsiI cuts
between
8016-8017
TfiI cuts
between
7928-7929
AccI ciuts
between
8360-8361
8386-8387
SprI site
between
8389-
8390
E3
8563-8564
NsiI cuts
between
8621-2862
AccI ciuts
between
8978-8979
AccI ciuts
between
9716-9717
XbaI cuts
between
9734-9735
9831-9832
E2
6K
9997-9998
E1
ATT at
11322-11324
3'NCR

Fig.1.

Fig.2a.

```
L1     CTAGAGAATTCT
           TCTTAAGAGATC

       XbaI   EcoRI   XbaI
```

Fig.2b.

```
L4A    CTAGTGACATGGTGAATTCACCATGTCA
           ACTGTACCACTTAAGTGGTACAGTGATC

       SpeI  START Kozak EcoRI Kozak START SpeI
               ^                              ^
       3 nt of 5' of E1                 3 nt of 5' of E1
```

Fig.2c.

```
L7L8   GATCCGTATACTCTAGAGCATG
            GCATATGAGATCTC

       BamHI   AccI   XbaI   SphI
```

Fig.2d.

```
L5L6   ATACTCCGGAGAACTGCGAGCAATGGTAAT
         TGAGGCCTCTTGACGCTCGTTACCATTAGATC

       AccI          3' Core          STOP XbaI
```

Fig.2e.

```
L10L13        TTAATCTAGAAGATGCA
         ACGTAATTAGATCTTCT

         NsiI STOP Xbal Start NsiI
```

Fig.2f.

```
L28L29 TGAGGGGCCATCCGCT AACAAGAAACCAGGCAAGAGACAGC..
           CCCCGGTAGGCGA TTGTTCTTTGGTCCGTTCTCTGTCG

       Bsu 36I       nt7735-7750--Λ--       nt7886-8024

                                     ..GCATGGTCATGAAATTGG
                                       CGTACCAGTACTTTAACCTTA

                                                        TfiI
```

Fig.2g.

```
L30L31   ATACTCCGGAGAACTGCGAGCAATGGTCACTAGTA
           TGAGGCCTCTTGACGCTCGTTACCAGTGATCATTGCA
                                          --Λ--
         AccI          Core                  E3  SpeI  HindIII
```

Fig.2h.

P2 GGCC*GGATC*CGGATGTTCCCGTTCCAGCC

GC Rich tail *BamHI* Start 5' Core

Fig.2i.

P3 GCGC*GGATCC*CCTCAGGTGGCGCG

GC Rich tail *BamHI* Bsu 36I Core

Fig.2j.

L20 GCGCCA *CTGAGG*GGCCATCCGC

GC Rich tail *Bsu 36I* 5' Nuclear targeting signal

Fig.2k.

L21 CGGG*GATTC*TGGTTTCTTGTTGGTCTTCTTCTTG

GC Rich tail *TfiI* 3' Nuclear targeting signal

Fig.3.

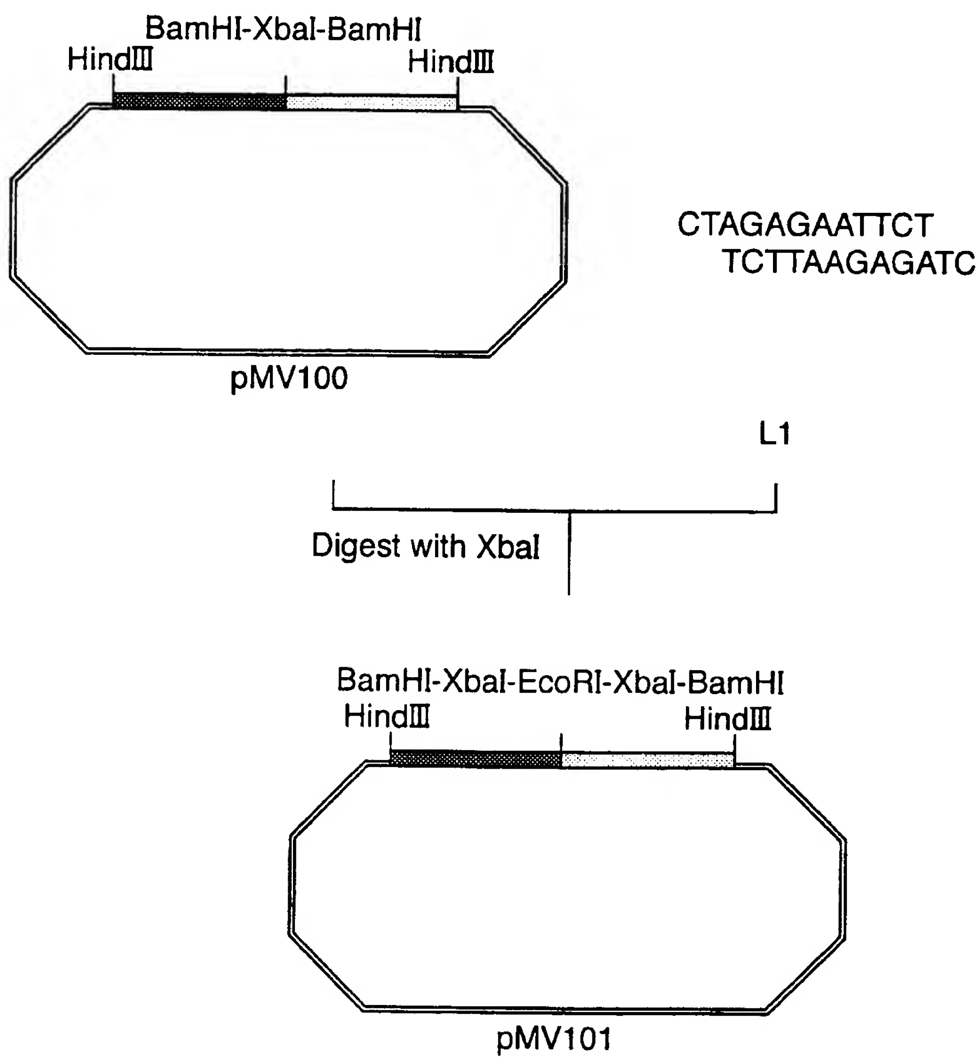

BamHI-XbaI-BamHI
HindIII
HindIII
pMV100
CTAGAGAATTCT
TCTTAAGAGATC
L1
Digest with XbaI
BamHI-XbaI-EcoRI-XbaI-BamHI
HindIII
HindIII
pMV101

Fig.4.

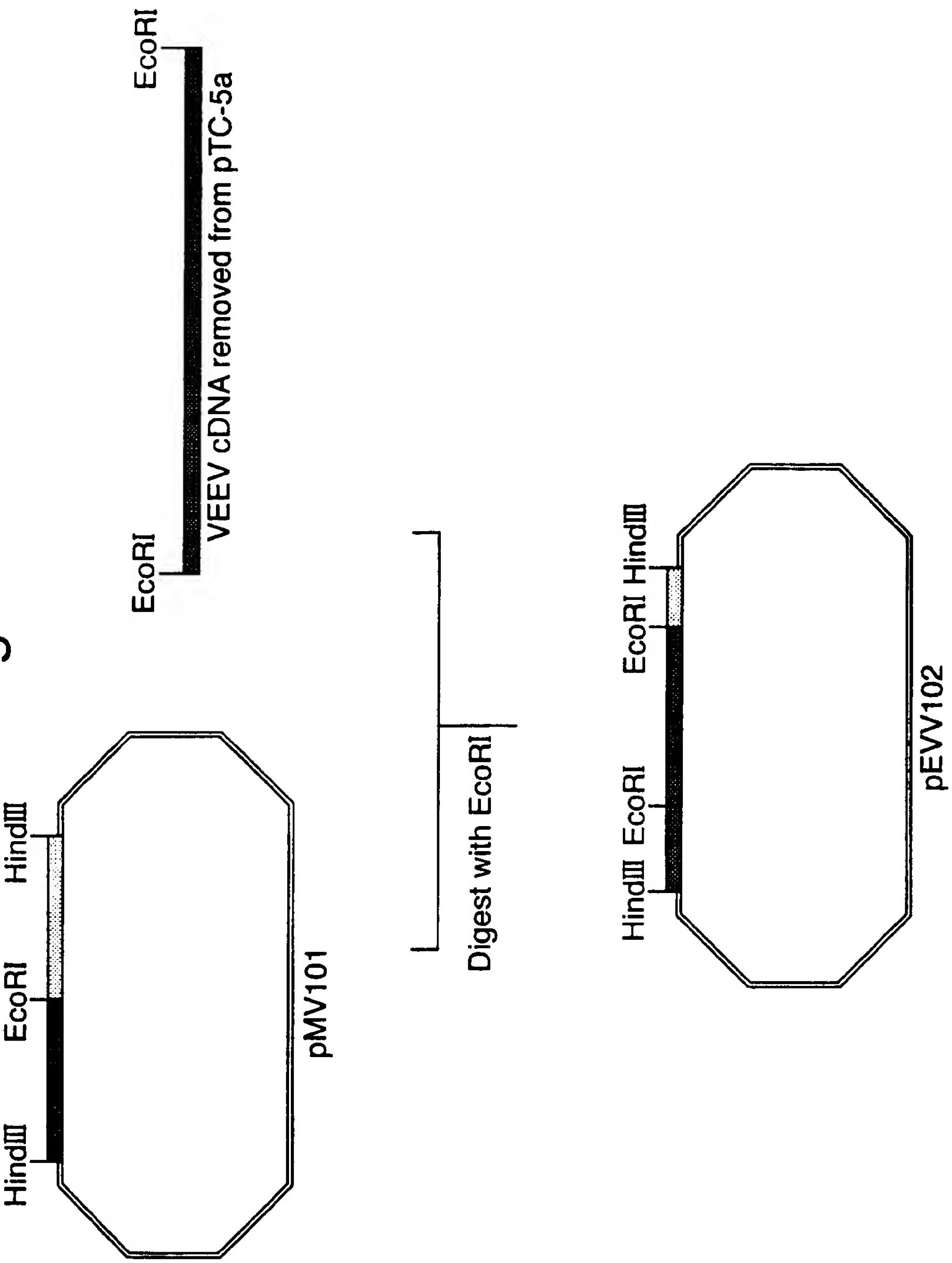
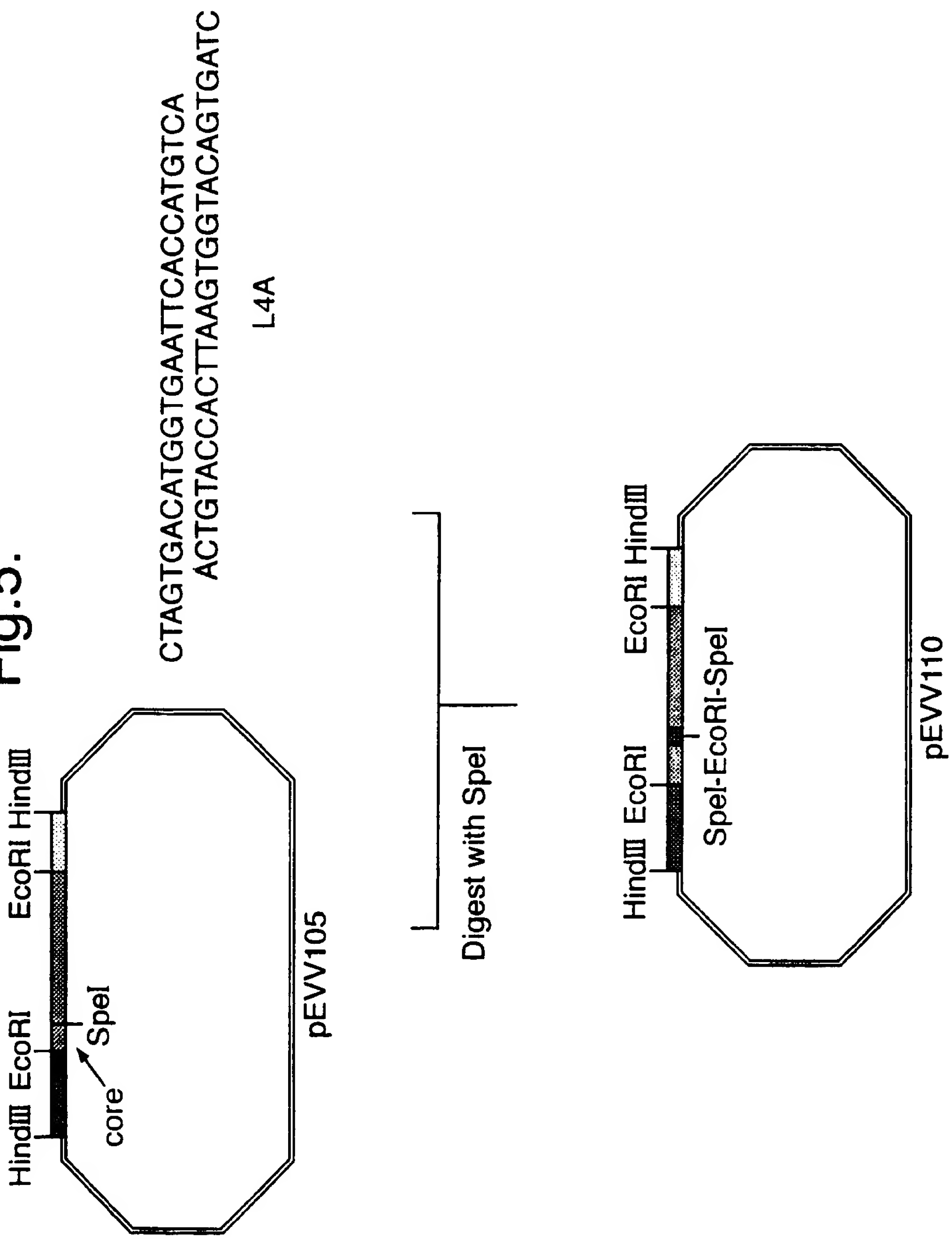

EcoRI
EcoRI
VEEV cDNA removed from pTC-5a
HindIII
EcoRI
HindIII
pMV101
Digest with EcoRI
HindIII
EcoRI
EcoRI
HindIII
pEVV102

Fig.5.


CTAGTGACATGGTGAATTCACCATGTCA
ACTGTACCACTTAAGTGGTACAGTGATC
L4A
HindIII EcoRI EcoRI HindIII
core
SpeI
pEVV105
Digest with SpeI
HindIII EcoRI EcoRI HindIII
SpeI-EcoRI-SpeI
pEVV110

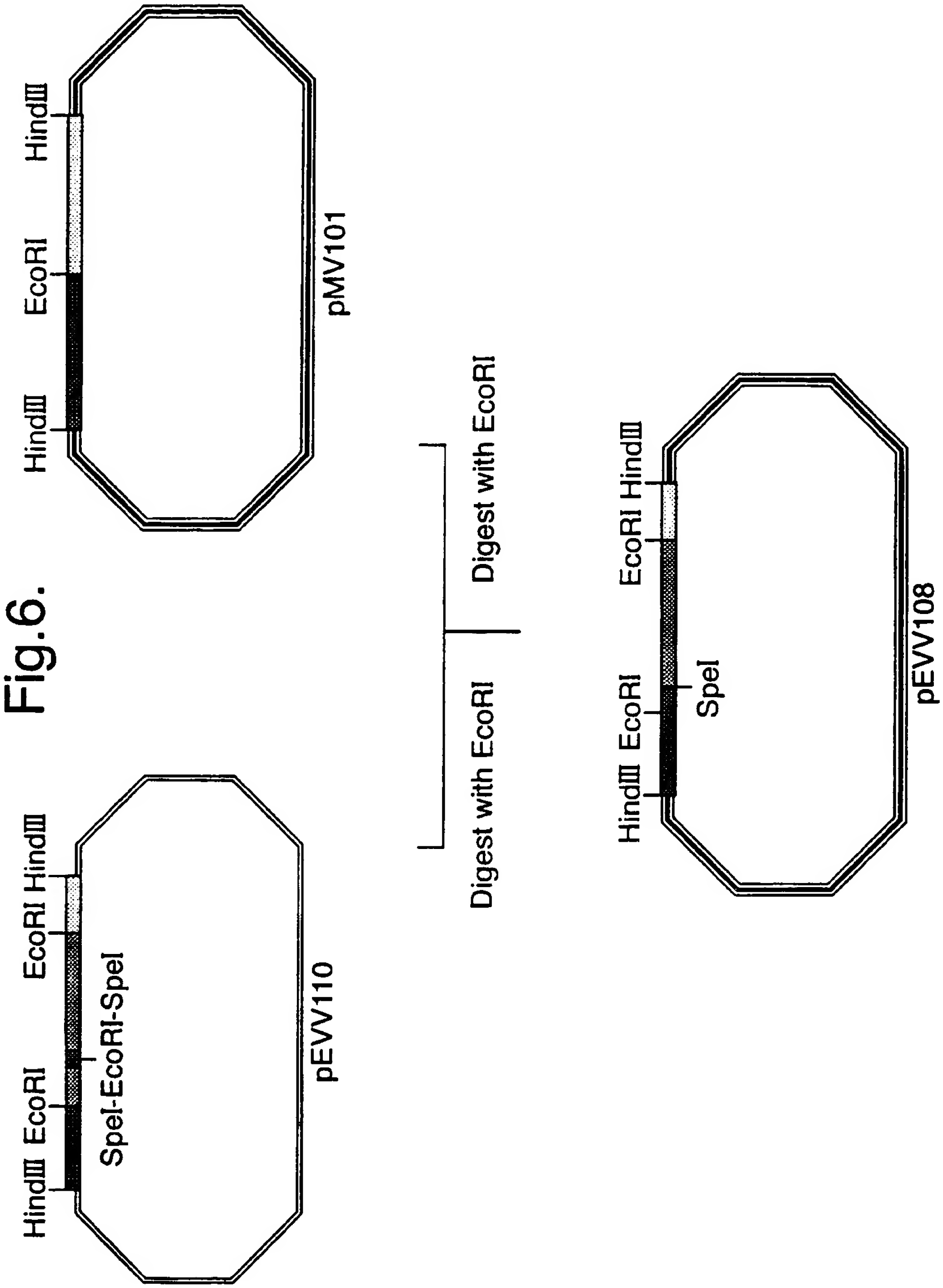

HindIII EcoRI HindIII
pMV101
Digest with EcoRI
Digest with EcoRI
HindIII EcoRI EcoRI HindIII
SpeI
pEVV108
HindIII EcoRI EcoRI HindIII
SpeI-EcoRI-SpeI
pEVV110

Fig.6.

Fig.7.

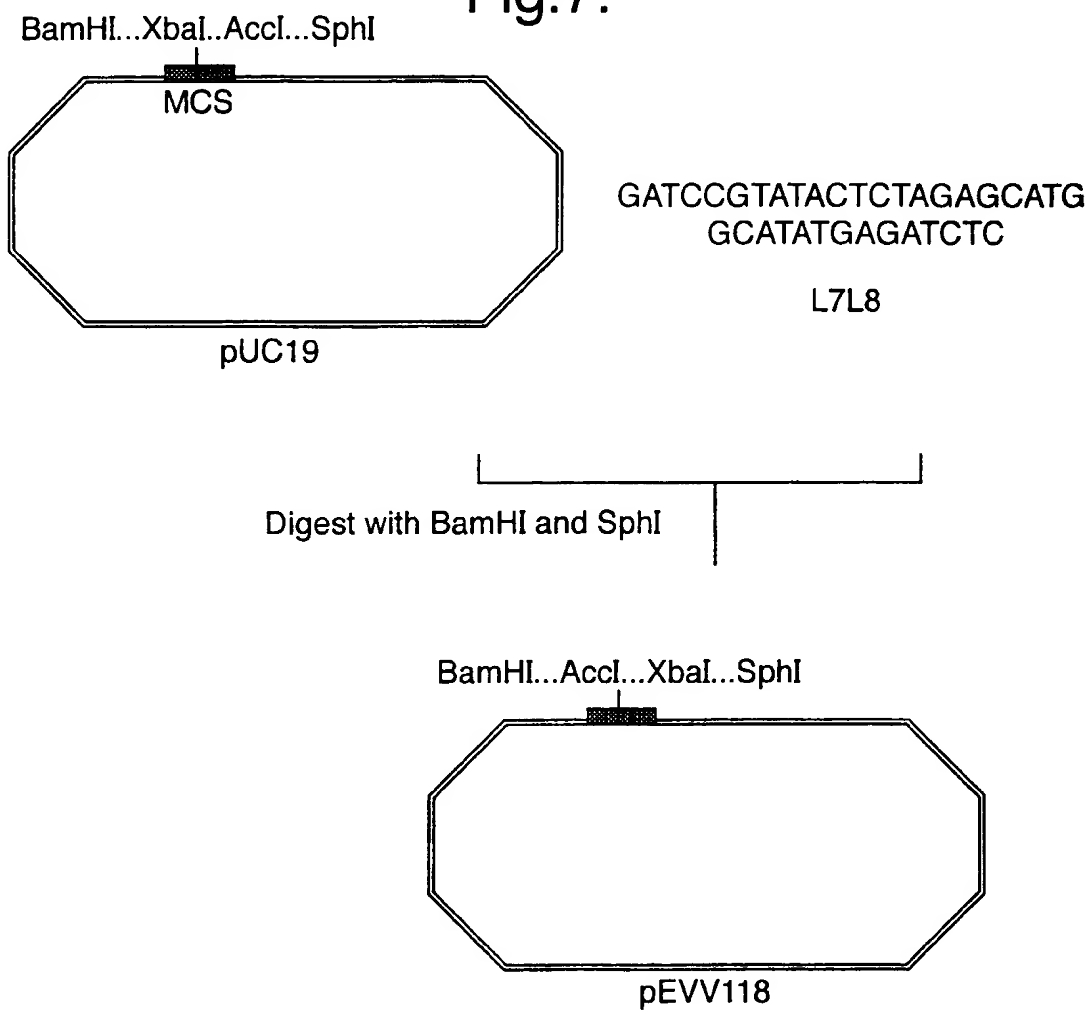

BamHI...XbaI..AccI...SphI
MCS
pUC19
GATCCGTATACTCTAGAGCATG
GCATATGAGATCTC
L7L8
Digest with BamHI and SphI
BamHI...AccI...XbaI...SphI
pEVV118

Fig.8.

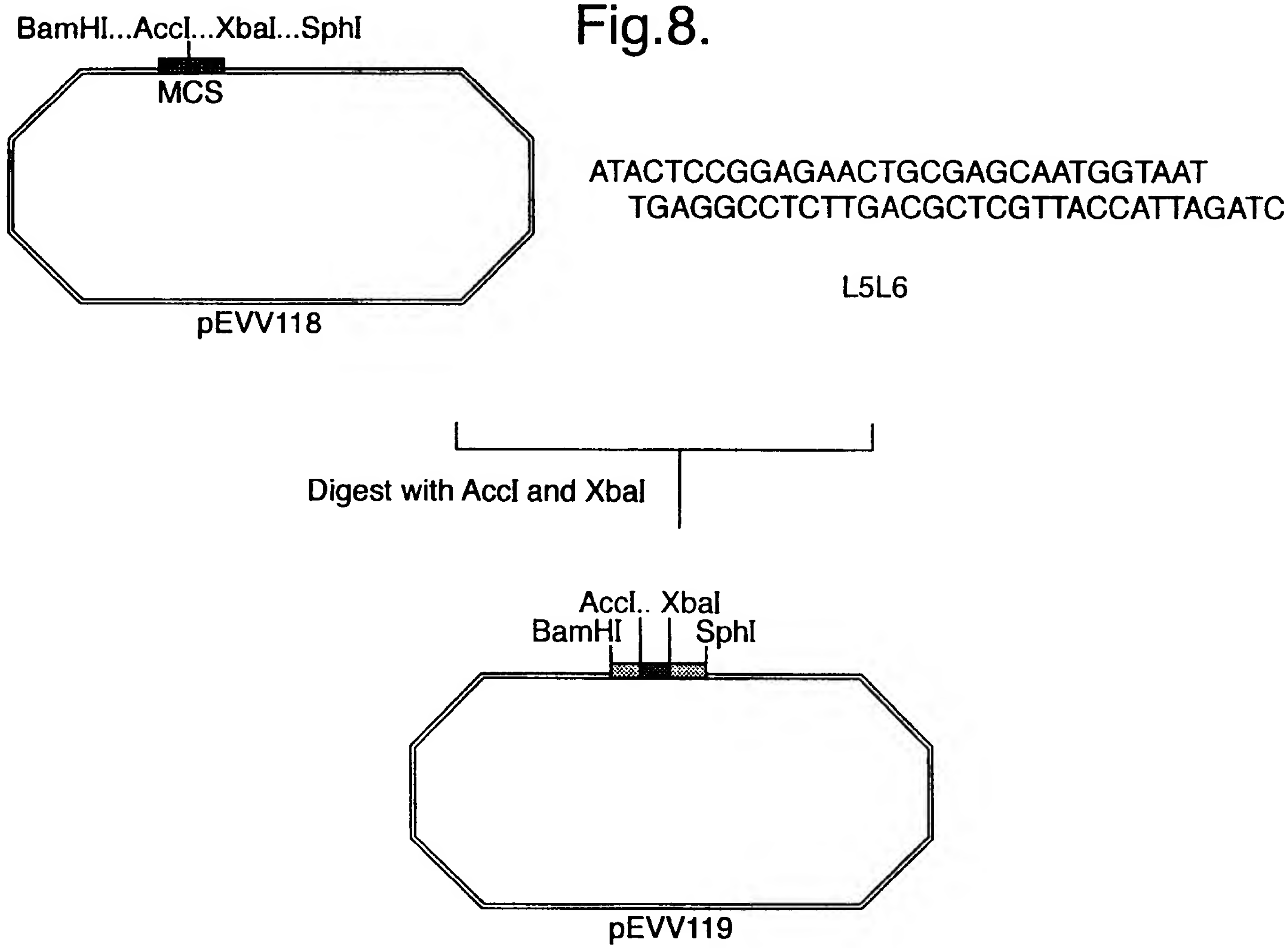

BamHI...AccI...XbaI...SphI
MCS
pEVV118
ATACTCCGGAGAACTGCGAGCAATGGTAAT
TGAGGCCTCTTGACGCTCGTTACCATTAGATC
L5L6
Digest with AccI and XbaI
AccI.. XbaI
BamHI
SphI
pEVV119

Fig.9.

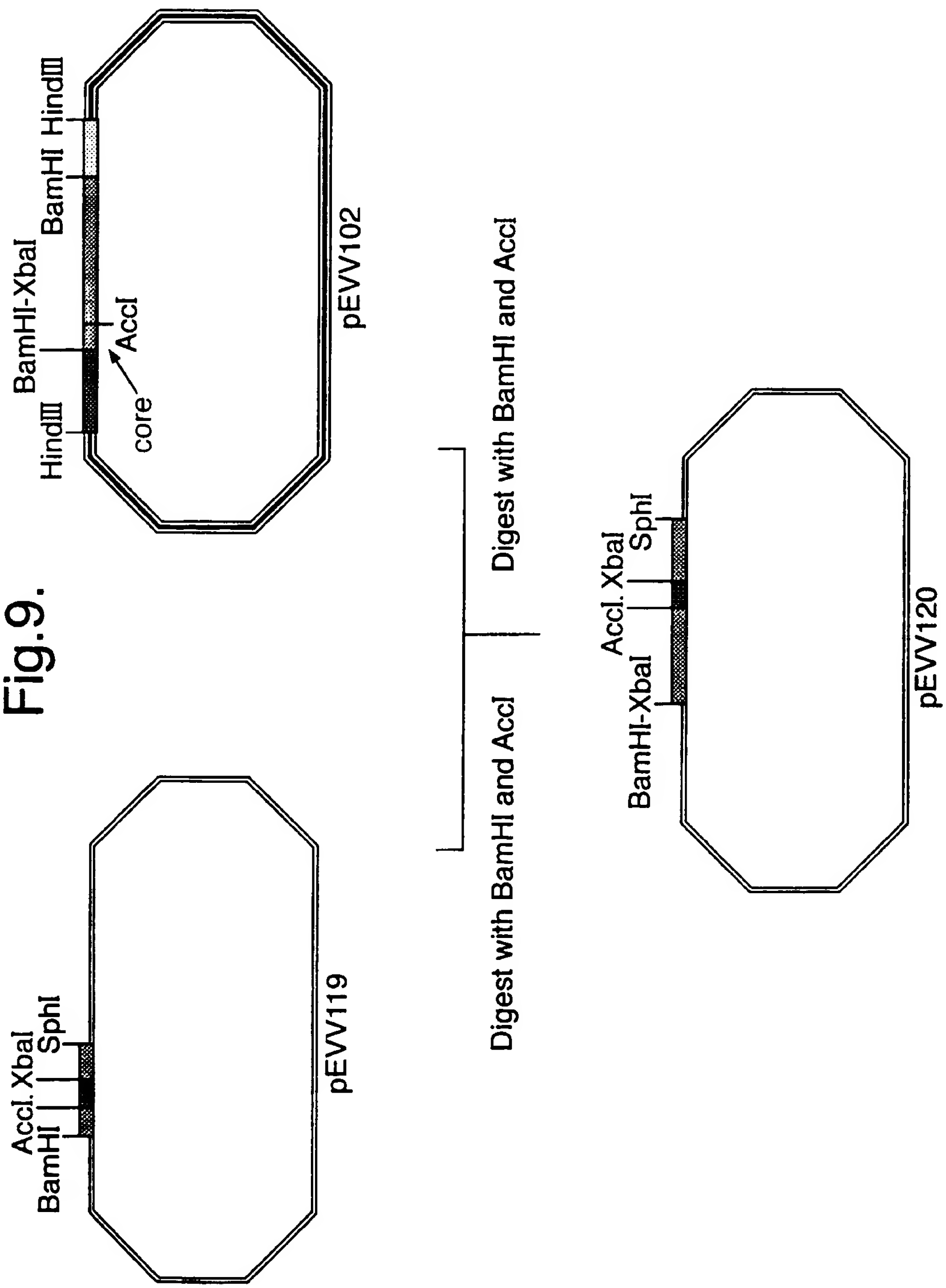

HindIII
BamHI-XbaI
BamHI
HindIII
core
AccI
pEVV102
Digest with BamHI and AccI
Digest with BamHI and AccI
BamHI
AccI. XbaI
SphI
pEVV119
BamHI-XbaI
AccI. XbaI
SphI
pEVV120

Fig.10.

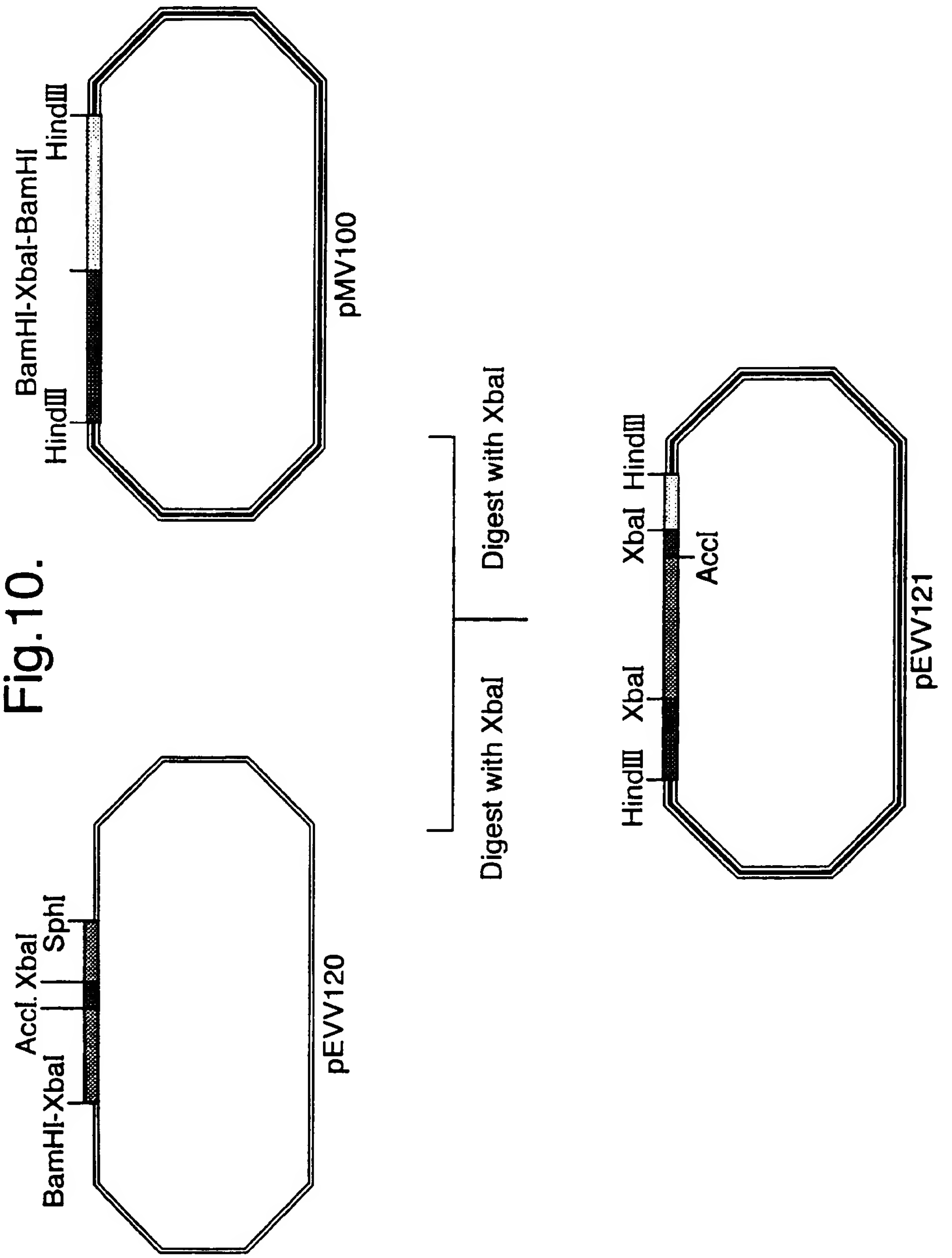
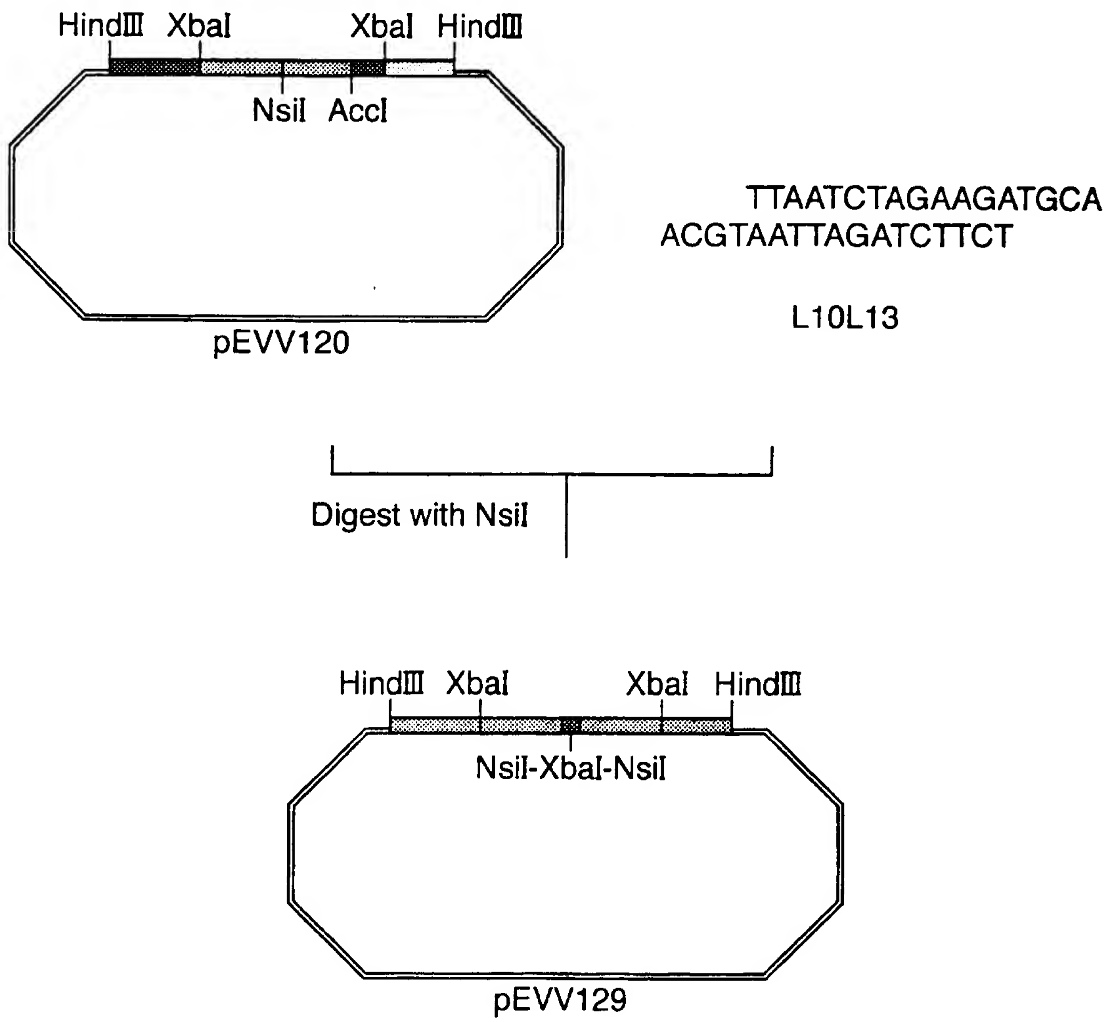

HindIII
BamHI-XbaI-BamHI
HindIII
pMV100
Digest with XbaI
Digest with XbaI
BamHI-XbaI
AccI. XbaI
SphI
pEVV120
HindIII
XbaI
XbaI
AccI
HindIII
pEVV121

Fig.11.


HindIII
XbaI
XbaI
HindIII
NsiI
AccI
pEVV120
TTAATCTAGAAGATGCA
ACGTAATTAGATCTTCT
L10L13
Digest with NsiI
HindIII
XbaI
XbaI
HindIII
NsiI-XbaI-NsiI
pEVV129

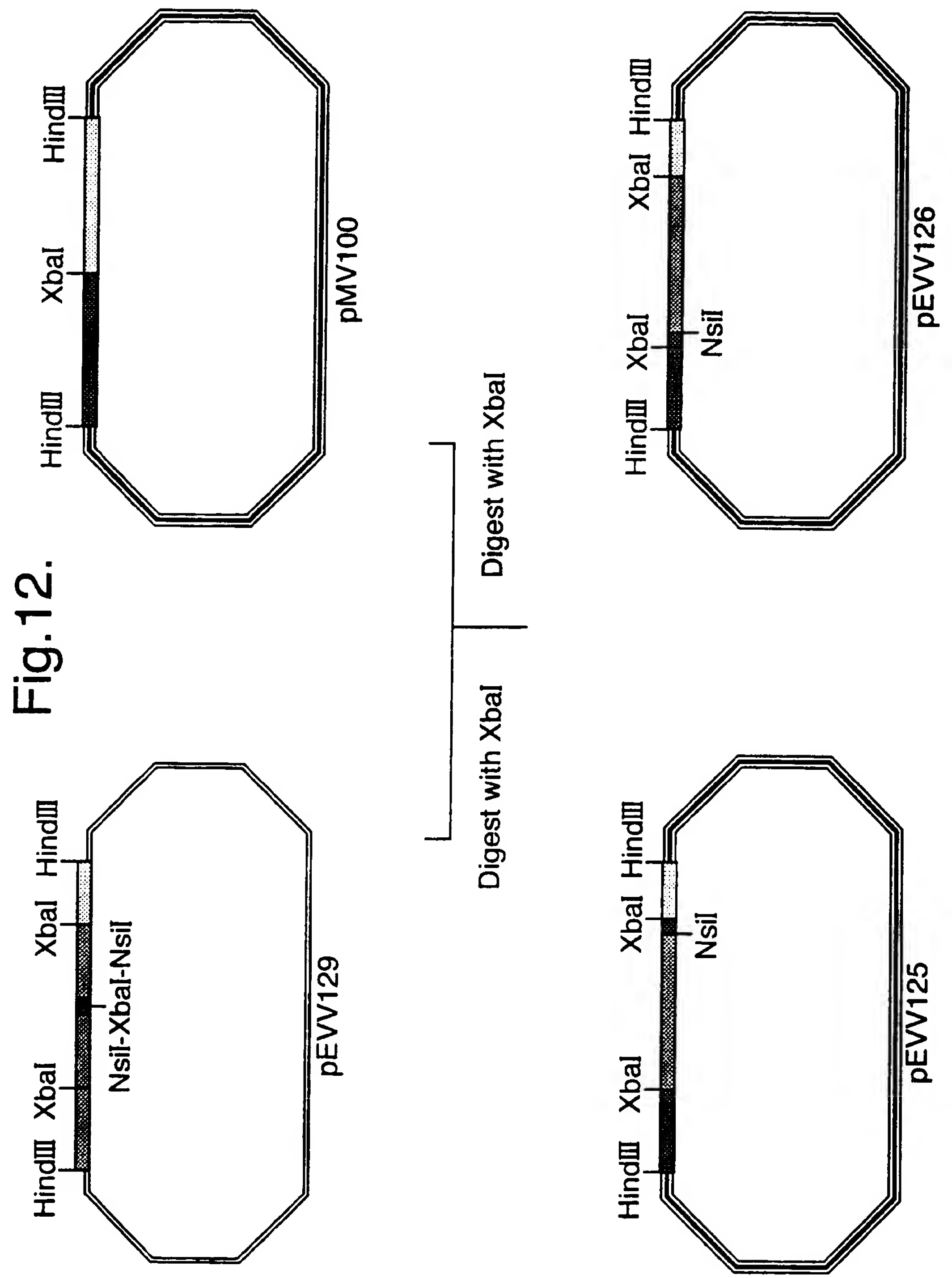

HindIII
XbaI
HindIII
pMV100
HindIII
XbaI
NsiI
XbaI
HindIII
pEVV126
Digest with XbaI
Digest with XbaI
HindIII
XbaI
NsiI-XbaI-NsiI
XbaI
HindIII
pEVV129
HindIII
XbaI
NsiI
XbaI
HindIII
pEVV125

Fig.12.

Fig.13.

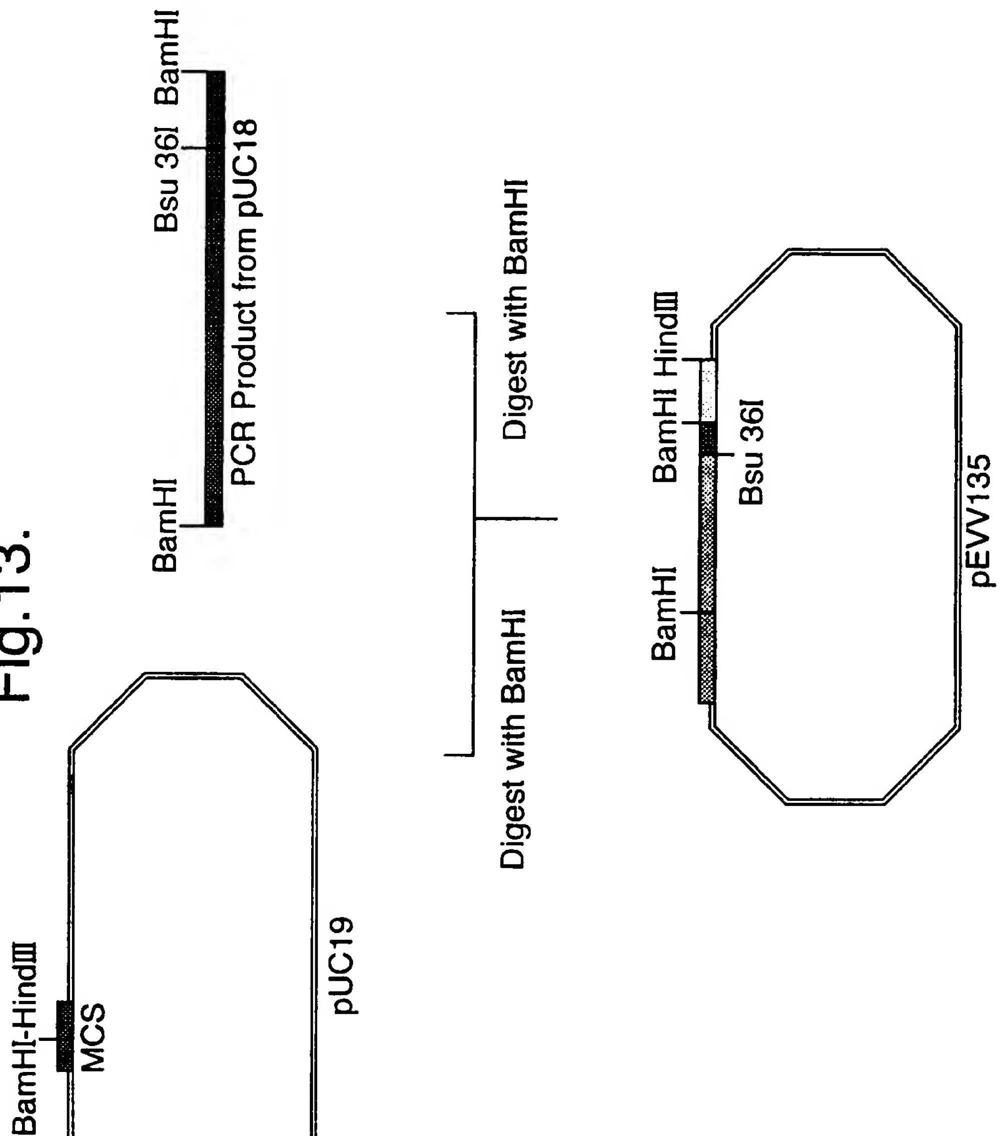

BamHI
Bsu 36I
BamHI
PCR Product from pUC18
BamHI-HindIII
MCS
pUC19
Digest with BamHI
Digest with BamHI
BamHI
BamHI
HindIII
Bsu 36I
pEVV135

Fig.14.

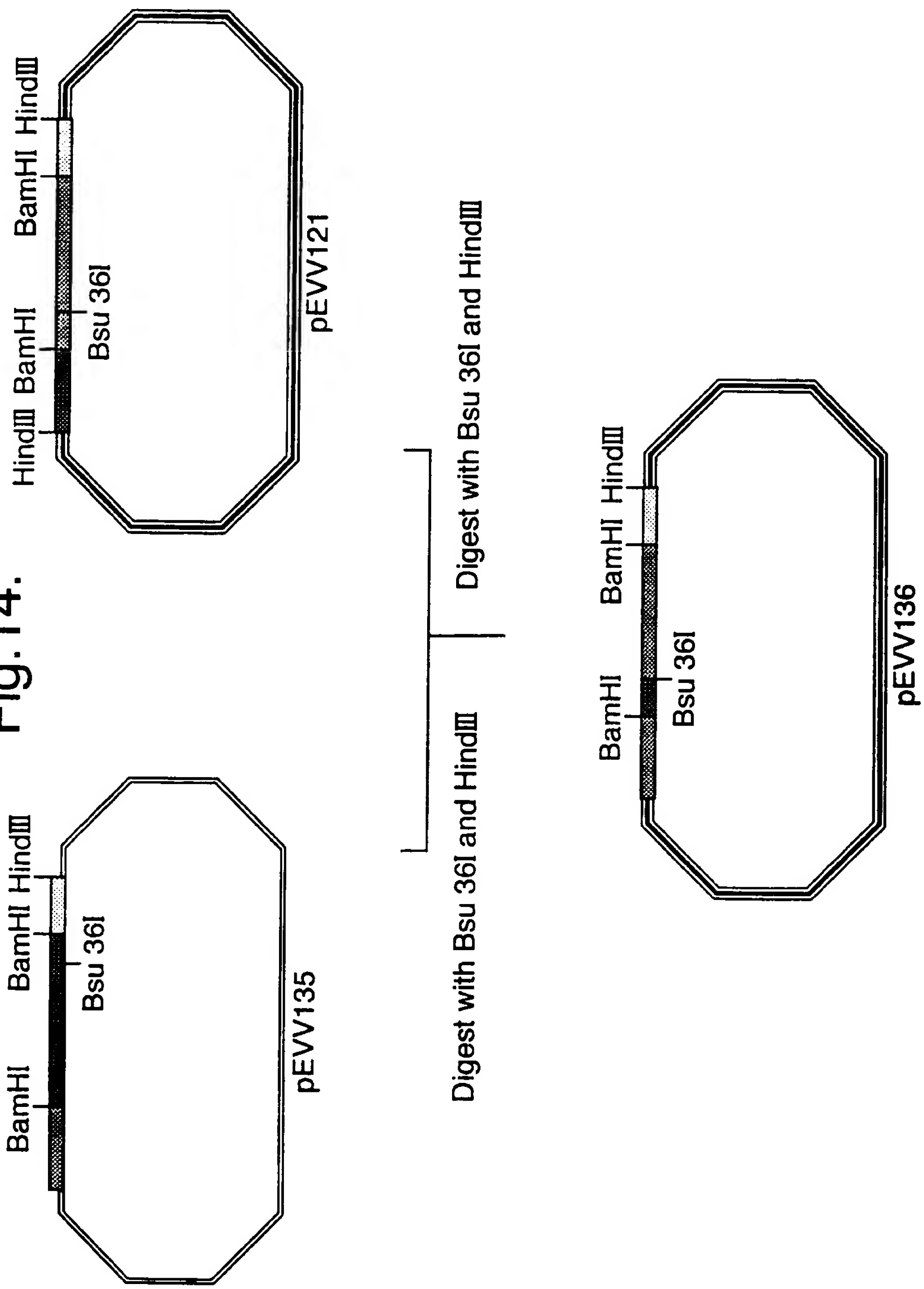

HindIII BamHI BamHI HindIII
Bsu 36I
pEVV121
Digest with Bsu 36I and HindIII
BamHI BamHI HindIII
Bsu 36I
pEVV135
Digest with Bsu 36I and HindIII
BamHI BamHI HindIII
Bsu 36I
pEVV136

Fig.15.

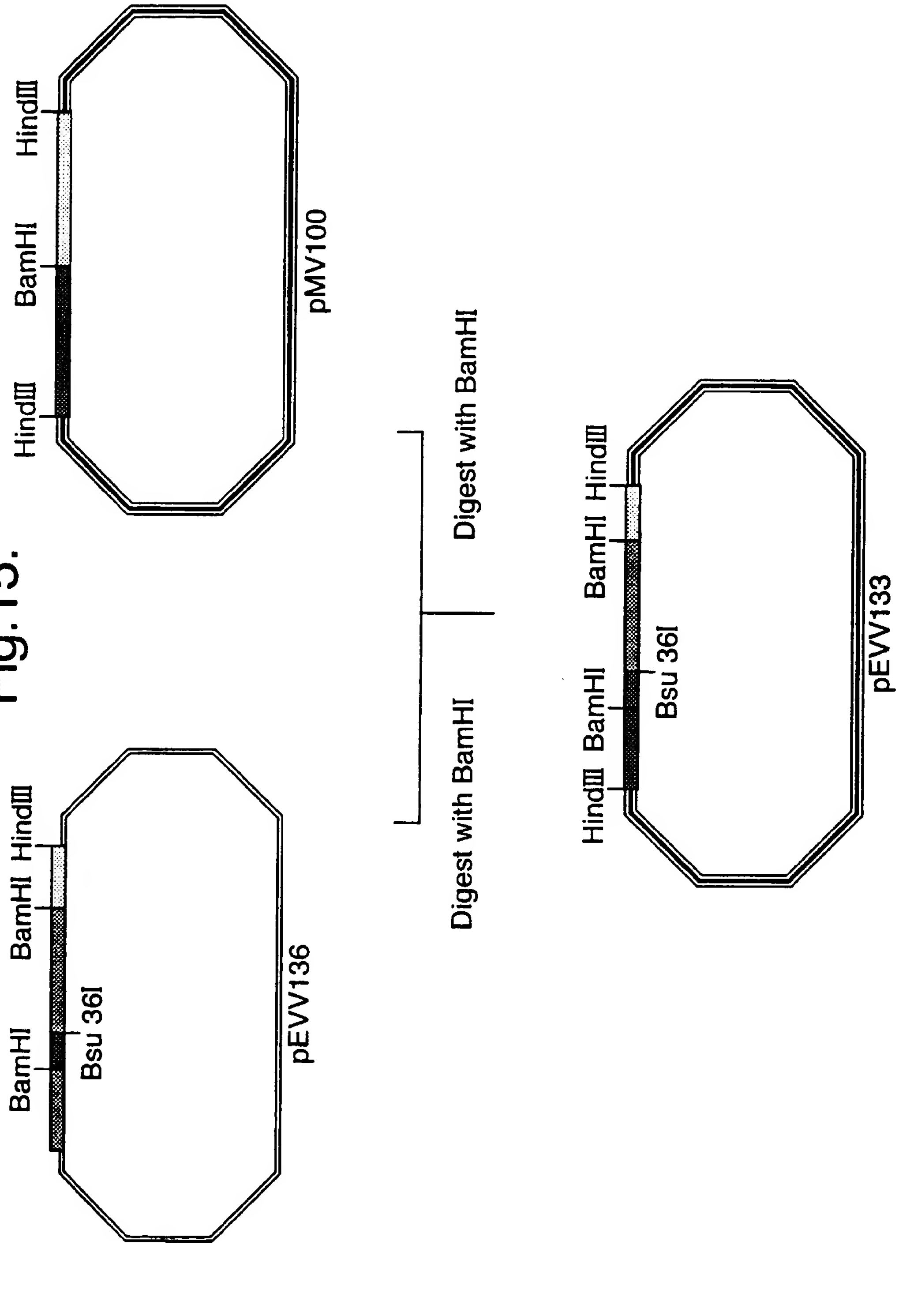

HindIII
BamHI
HindIII
pMV100
Digest with BamHI
Digest with BamHI
HindIII
BamHI
Bsu 36I
BamHI
HindIII
pEVV133
BamHI
Bsu 36I
BamHI
HindIII
pEVV136

Fig.16.

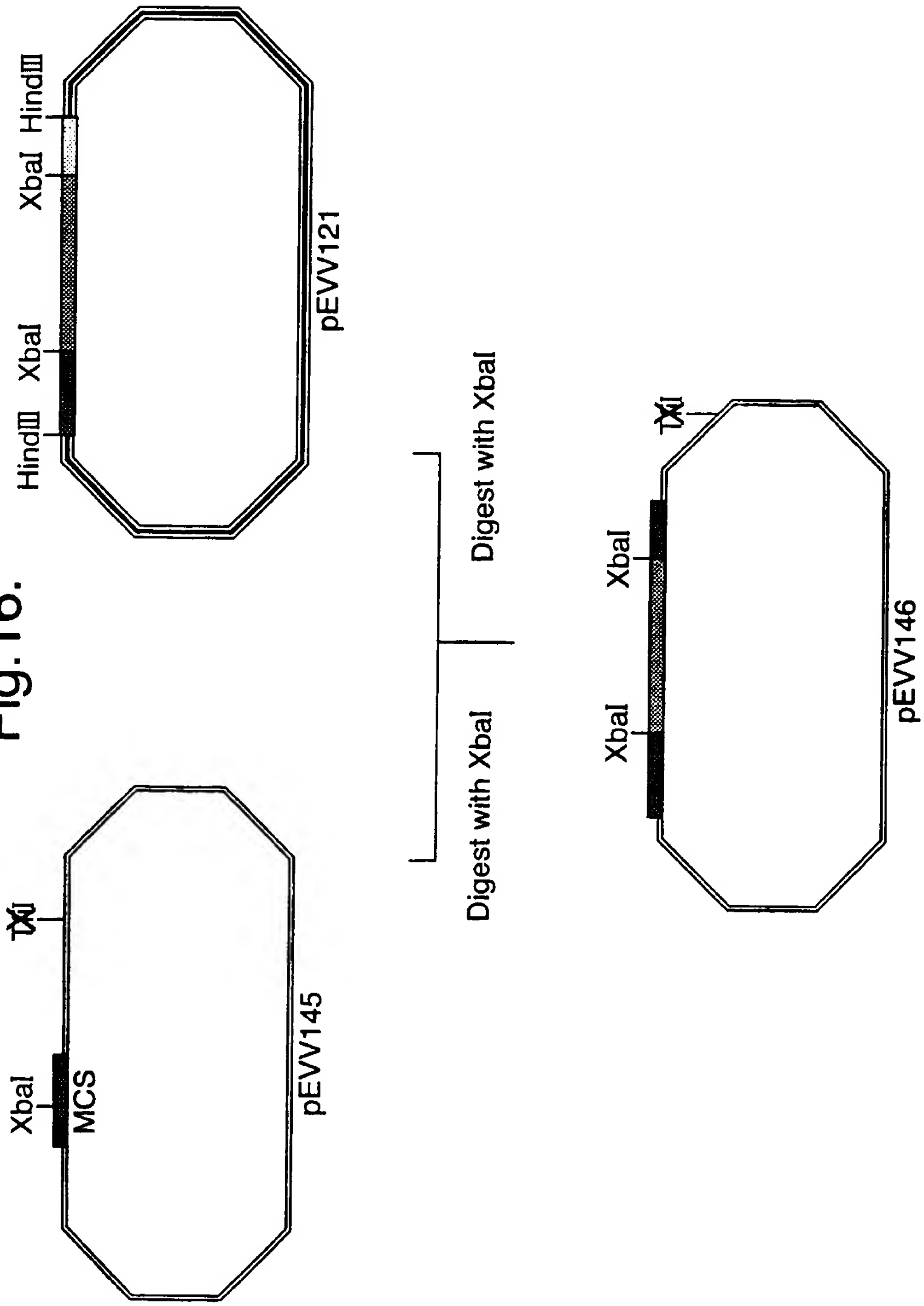
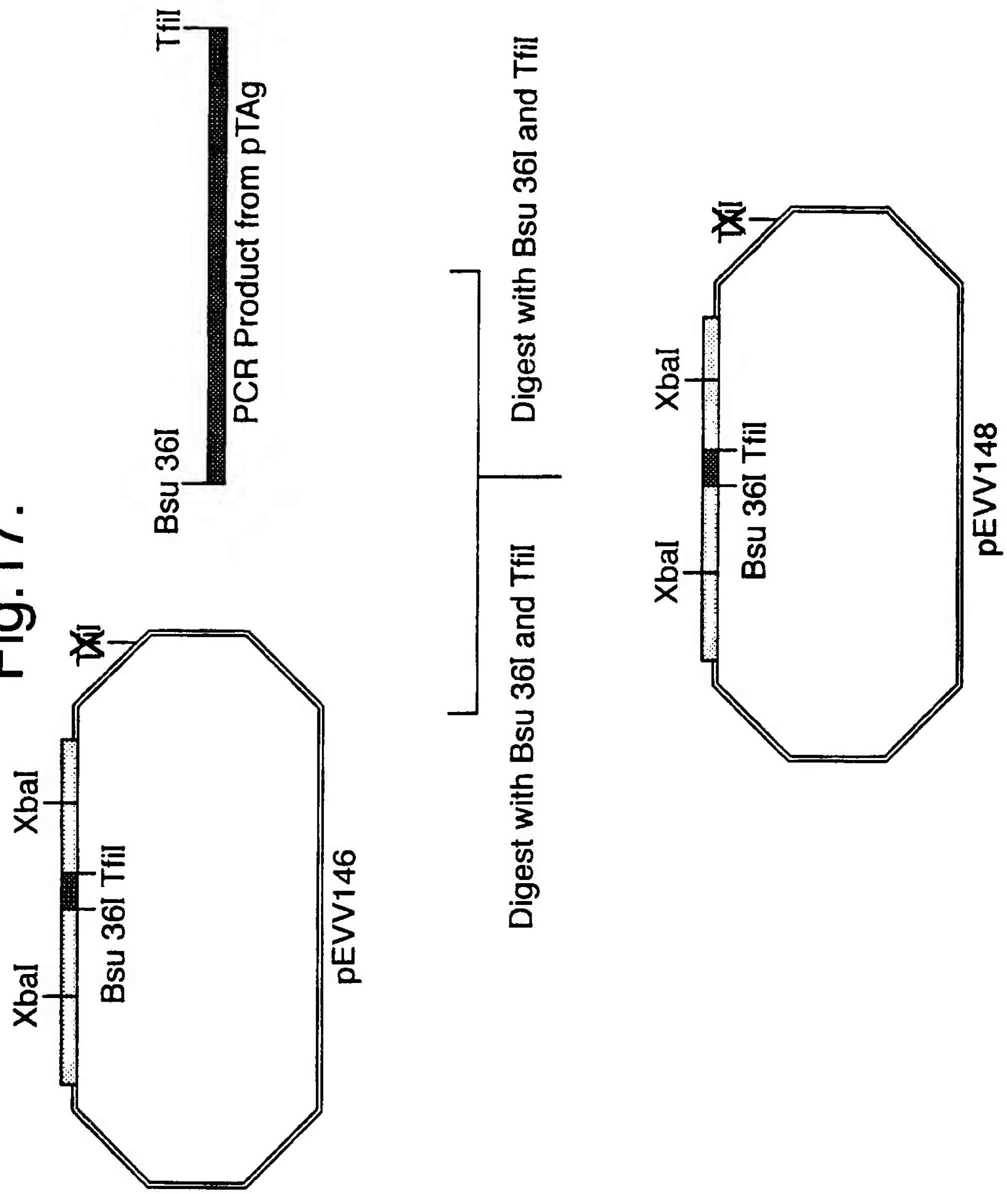

HindIII
XbaI
XbaI
HindIII
pEVV121
Digest with XbaI
Digest with XbaI
XbaI
MCS
pEVV145
XbaI
XbaI
pEVV146

Fig.17.


TfiI
PCR Product from pTAg
Bsu 36I
Digest with Bsu 36I and TfiI
Digest with Bsu 36I and TfiI
XbaI
XbaI
Bsu 36I TfiI
pEVV146
XbaI
XbaI
Bsu 36I TfiI
pEVV148

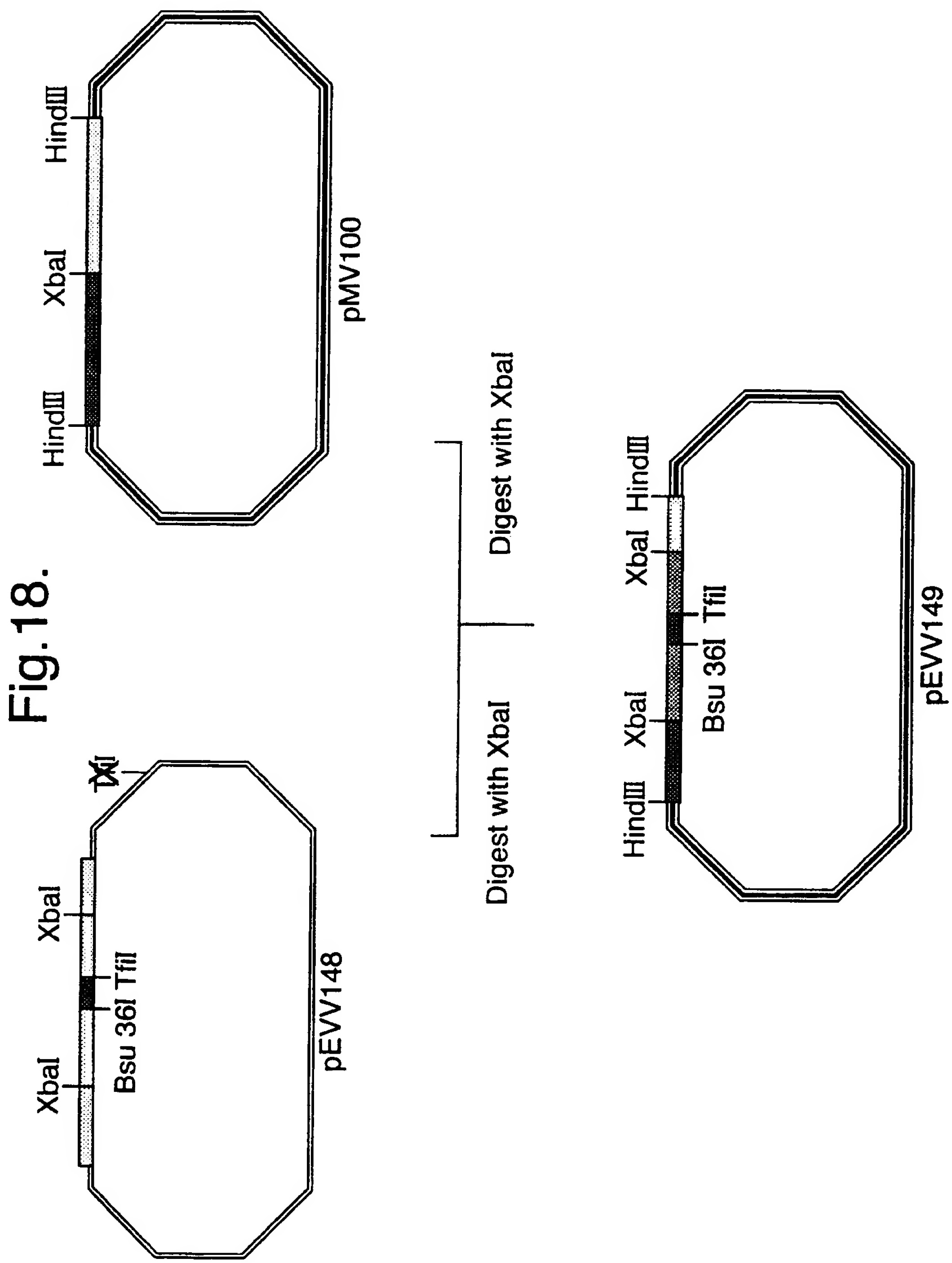

HindIII
XbaI
HindIII
pMV100
Digest with XbaI
Digest with XbaI
XbaI
Bsu 36I TfiI
XbaI
pEVV148
HindIII
XbaI
TfiI
Bsu 36I
XbaI
HindIII
pEVV149

Fig.18.

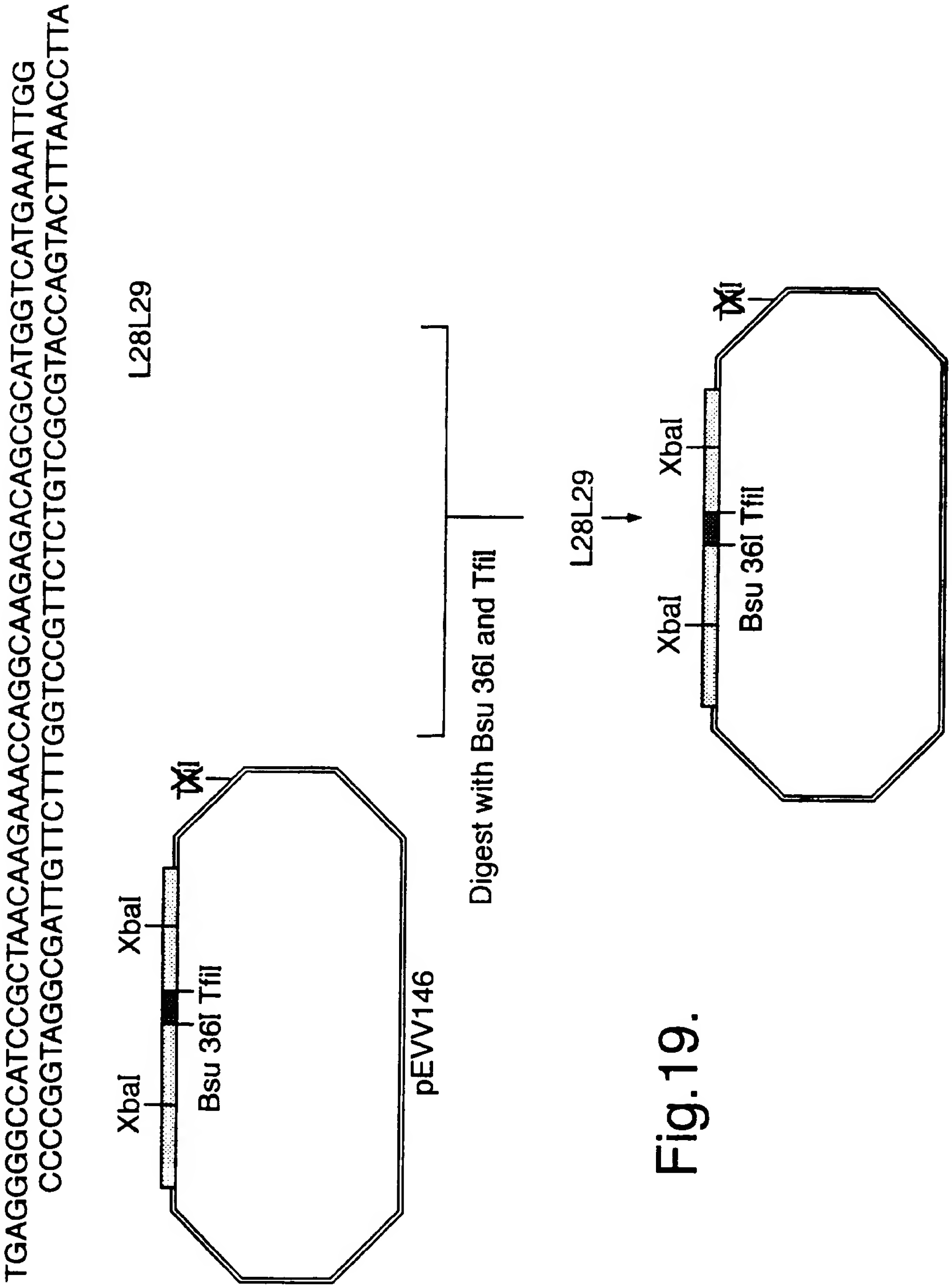

TGAGGGCCATCCGCTAACAAGAAACCAGGCAAGAGACAGCGCATGGTCATGAAATTGG
CCCCGGTAGGCGATTGTTCTTTGGTCCGTTCTCTGTCGCGTACCAGTACTTTAACCTTA
L28L29
XbaI
XbaI
Bsu 36I TfiI
XhoI
pEVV146
Digest with Bsu 36I and TfiI
L28L29
XbaI
XbaI
Bsu 36I TfiI
XhoI
pEVV151

Fig.19.

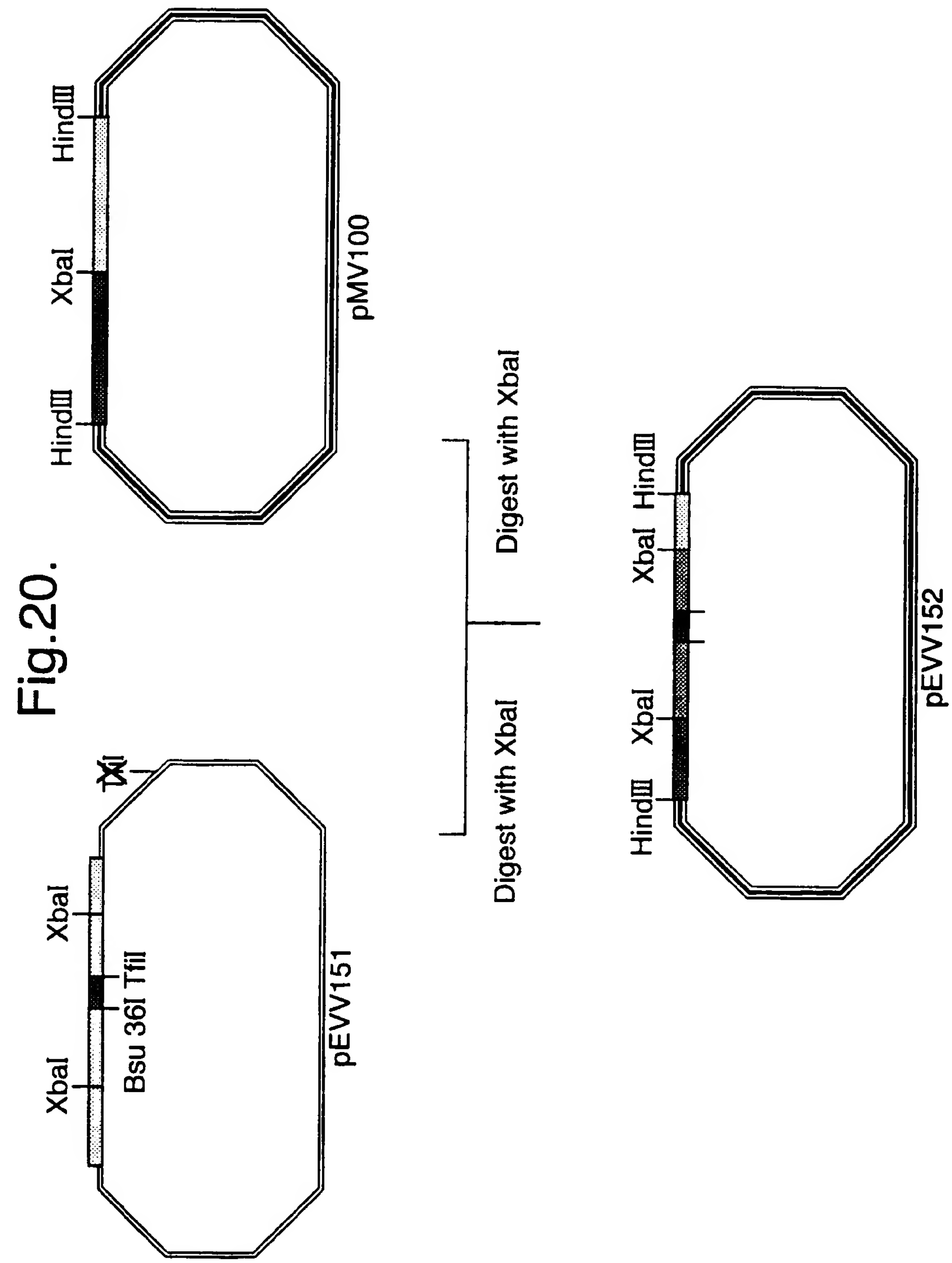

HindIII
XbaI
HindIII
pMV100
XbaI
XbaI
Bsu 36I TfiI
pEVV151
Digest with XbaI
Digest with XbaI
HindIII
XbaI
XbaI
HindIII
pEVV152

Fig.20.

Fig.21.

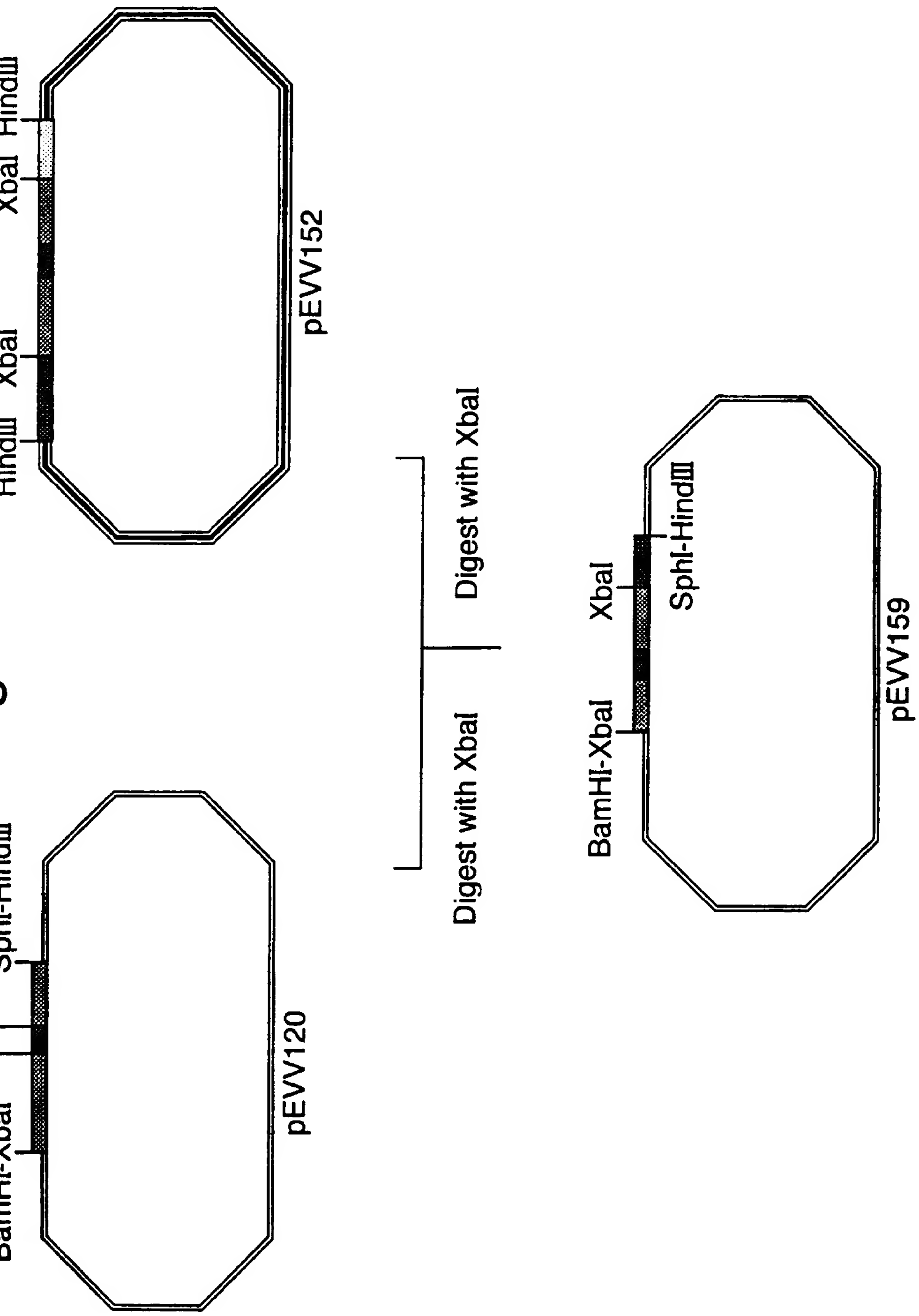
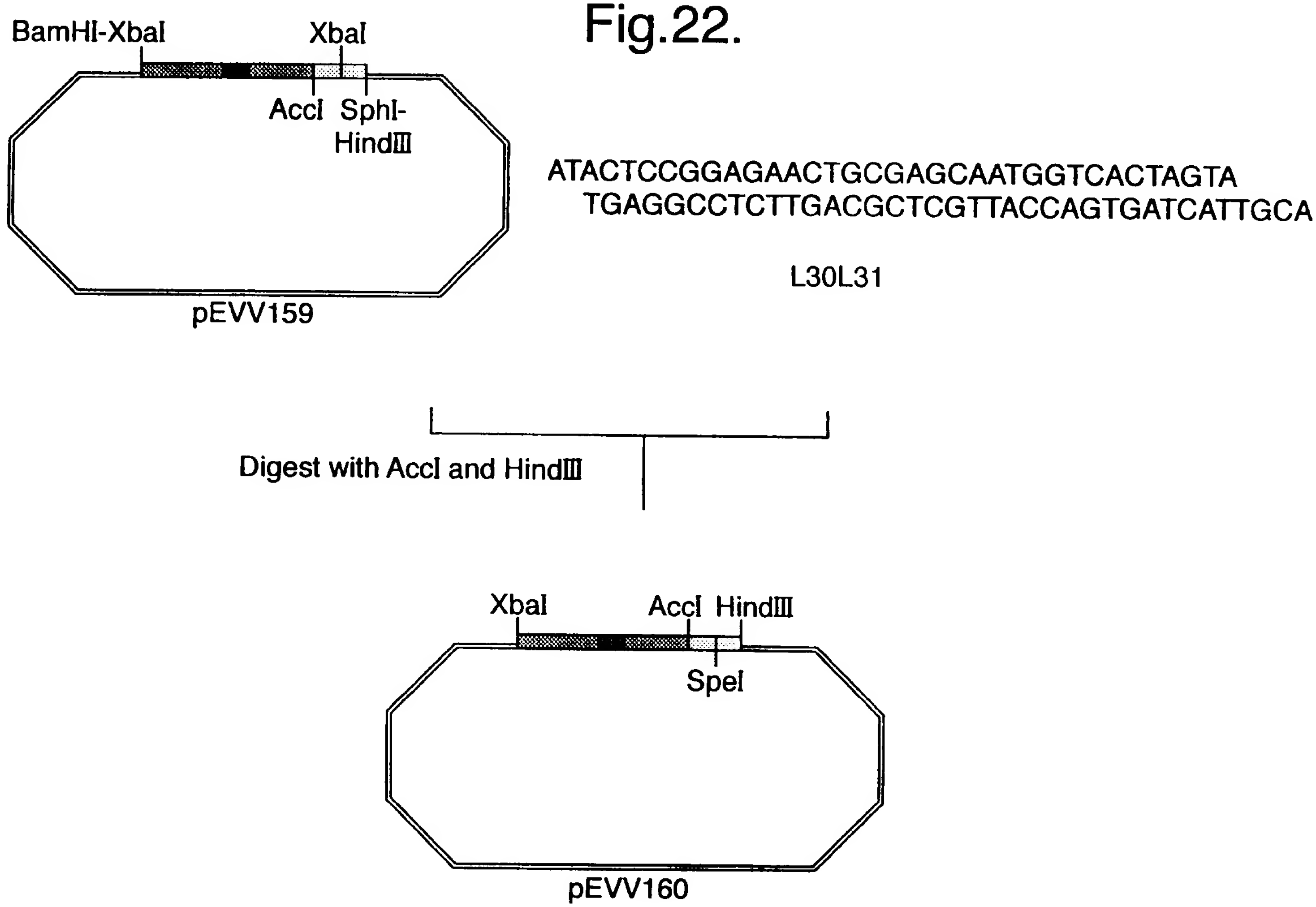

HindIII
XbaI
XbaI
HindIII
pEVV152
Digest with XbaI
Digest with XbaI
BamHI-XbaI
AccI.
XbaI
SphI-HindIII
pEVV120
BamHI-XbaI
XbaI
SphI-HindIII
pEVV159

Fig.22.


BamHI-XbaI
XbaI
AccI
SphI-HindIII
pEVV159
ATACTCCGGAGAACTGCGAGCAATGGTCACTAGTA
TGAGGCCTCTTGACGCTCGTTACCAGTGATCATTGCA
L30L31
Digest with AccI and HindIII
XbaI
AccI
HindIII
SpeI
pEVV160

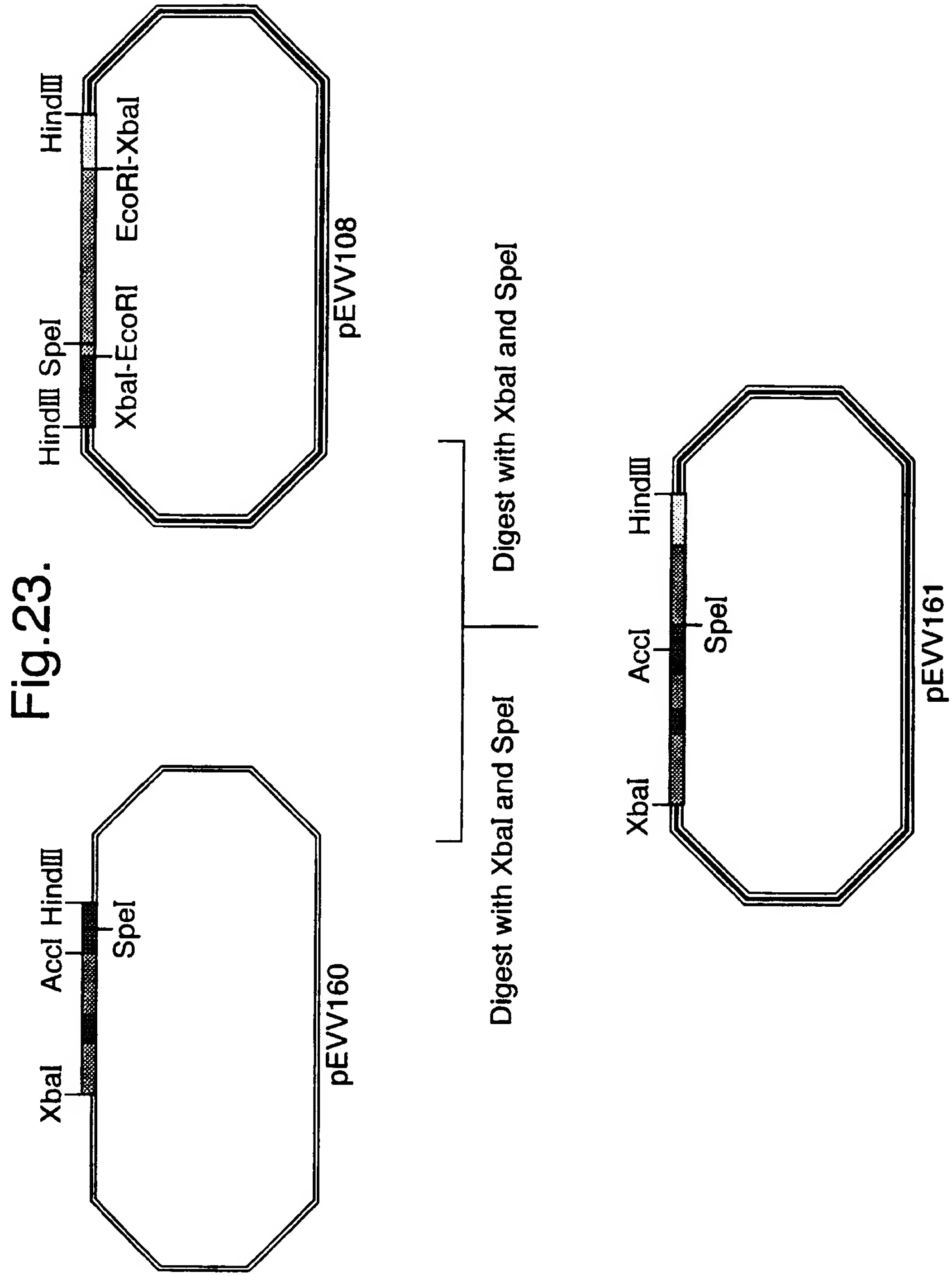

HindIII
EcoRI-XbaI
XbaI-EcoRI
HindIII SpeI
pEVV108
Digest with XbaI and SpeI
Digest with XbaI and SpeI
XbaI
AccI HindIII
SpeI
pEVV160
HindIII
SpeI
AccI
XbaI
pEVV161

Fig.23.

Fig.24.

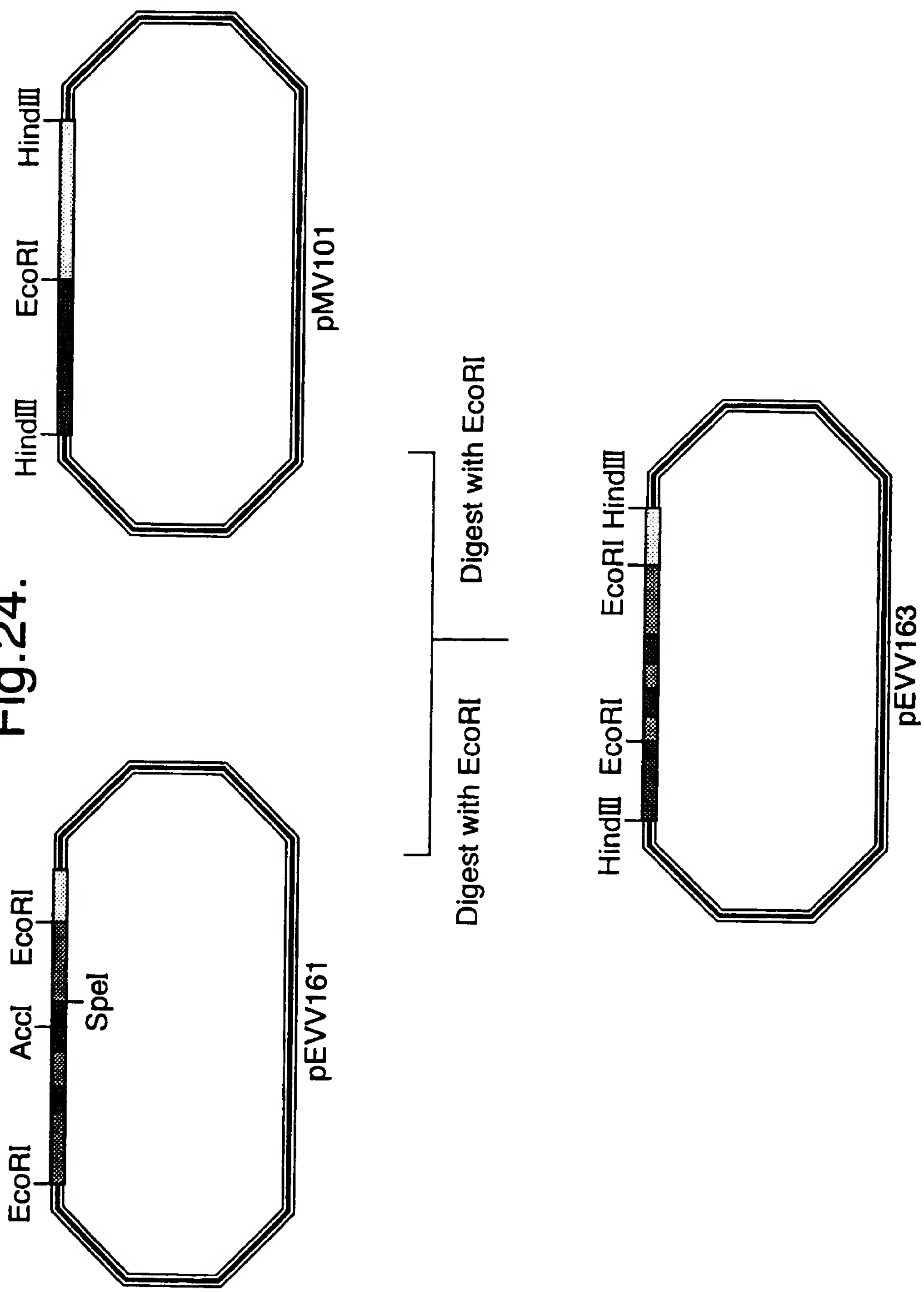

HindIII
EcoRI
HindIII
pMV101
Digest with EcoRI
Digest with EcoRI
EcoRI
AccI
SpeI
EcoRI
pEVV161
HindIII
EcoRI
EcoRI
HindIII
pEVV163

Fig.25.

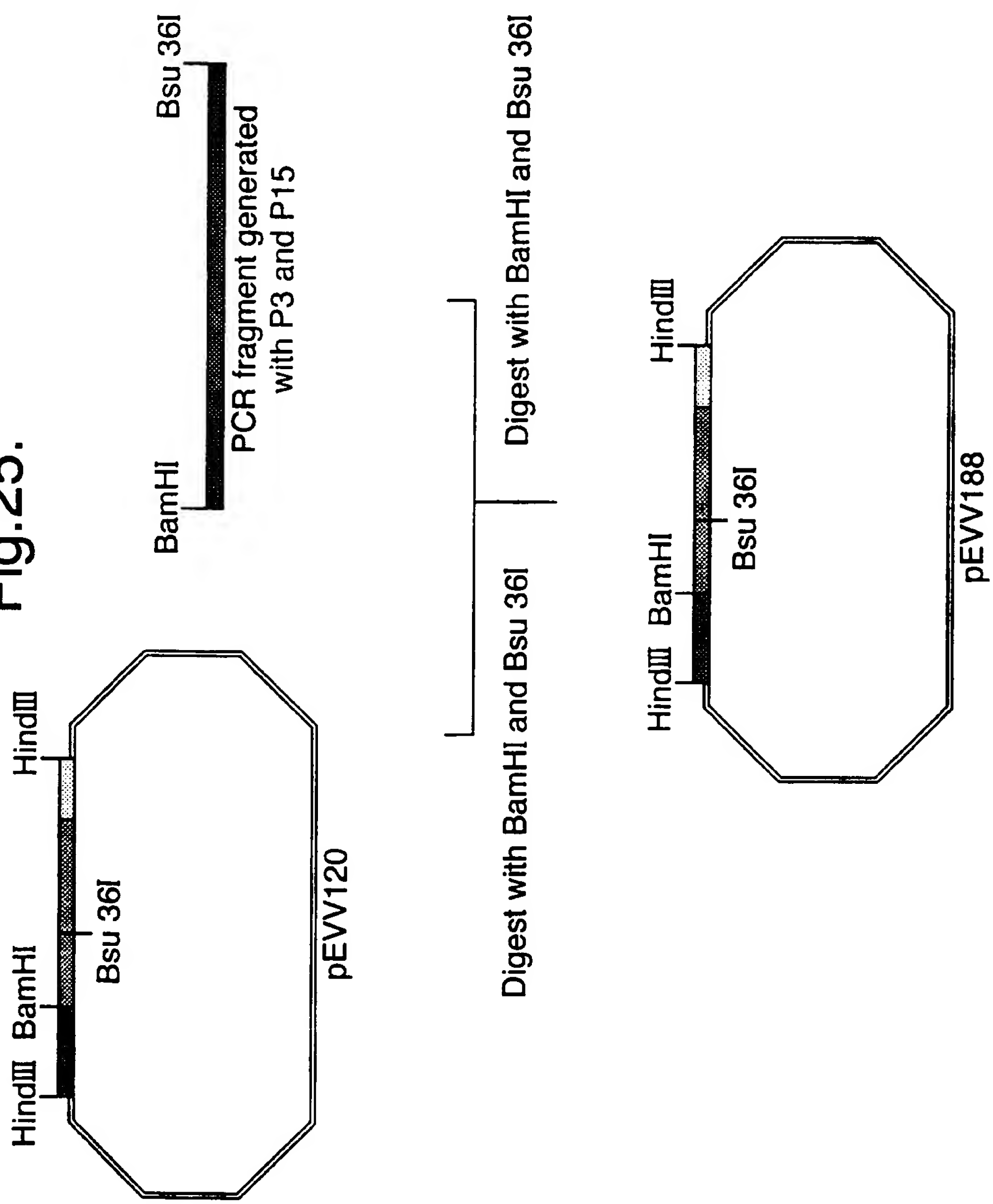
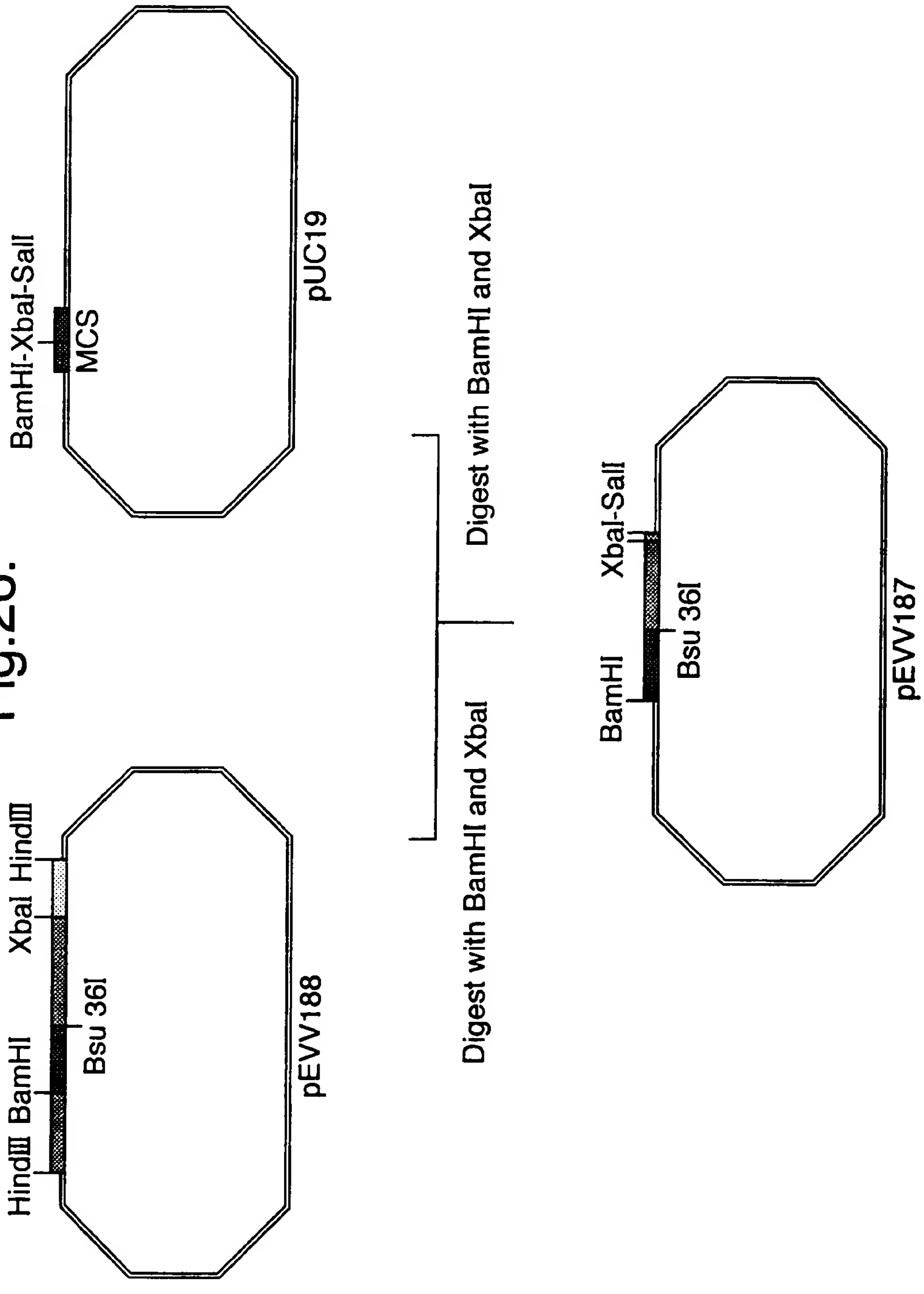

BamHI
Bsu 36I
PCR fragment generated with P3 and P15
HindIII
BamHI
Bsu 36I
HindIII
pEVV120
Digest with BamHI and Bsu 36I
Digest with BamHI and Bsu 36I
HindIII
BamHI
Bsu 36I
HindIII
pEVV188

Fig.26.


BamHI-XbaI-SalI
MCS
pUC19
Digest with BamHI and XbaI
HindIII BamHI XbaI HindIII
Bsu 36I
pEVV188
Digest with BamHI and XbaI
XbaI-SalI
BamHI
Bsu 36I
pEVV187

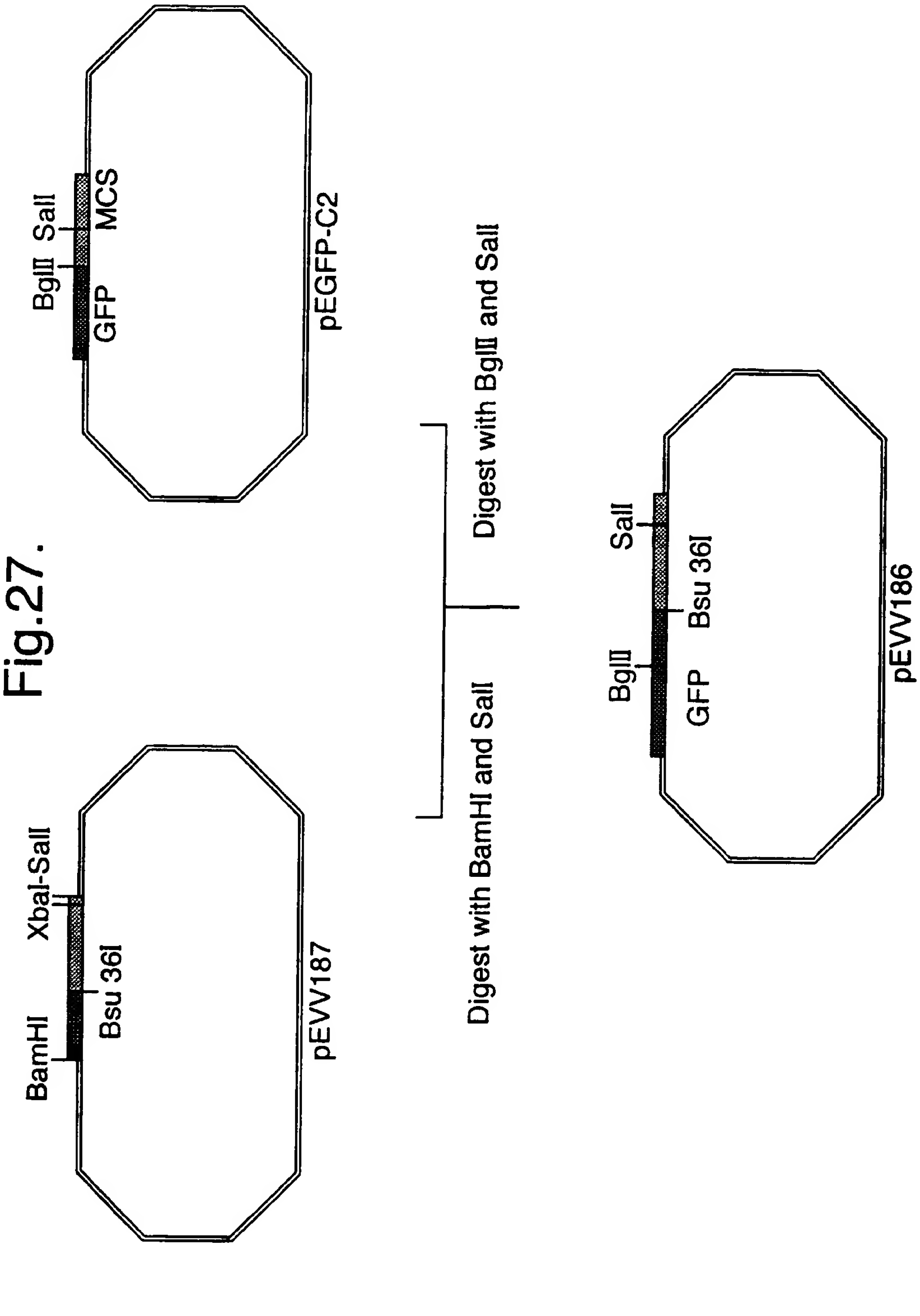
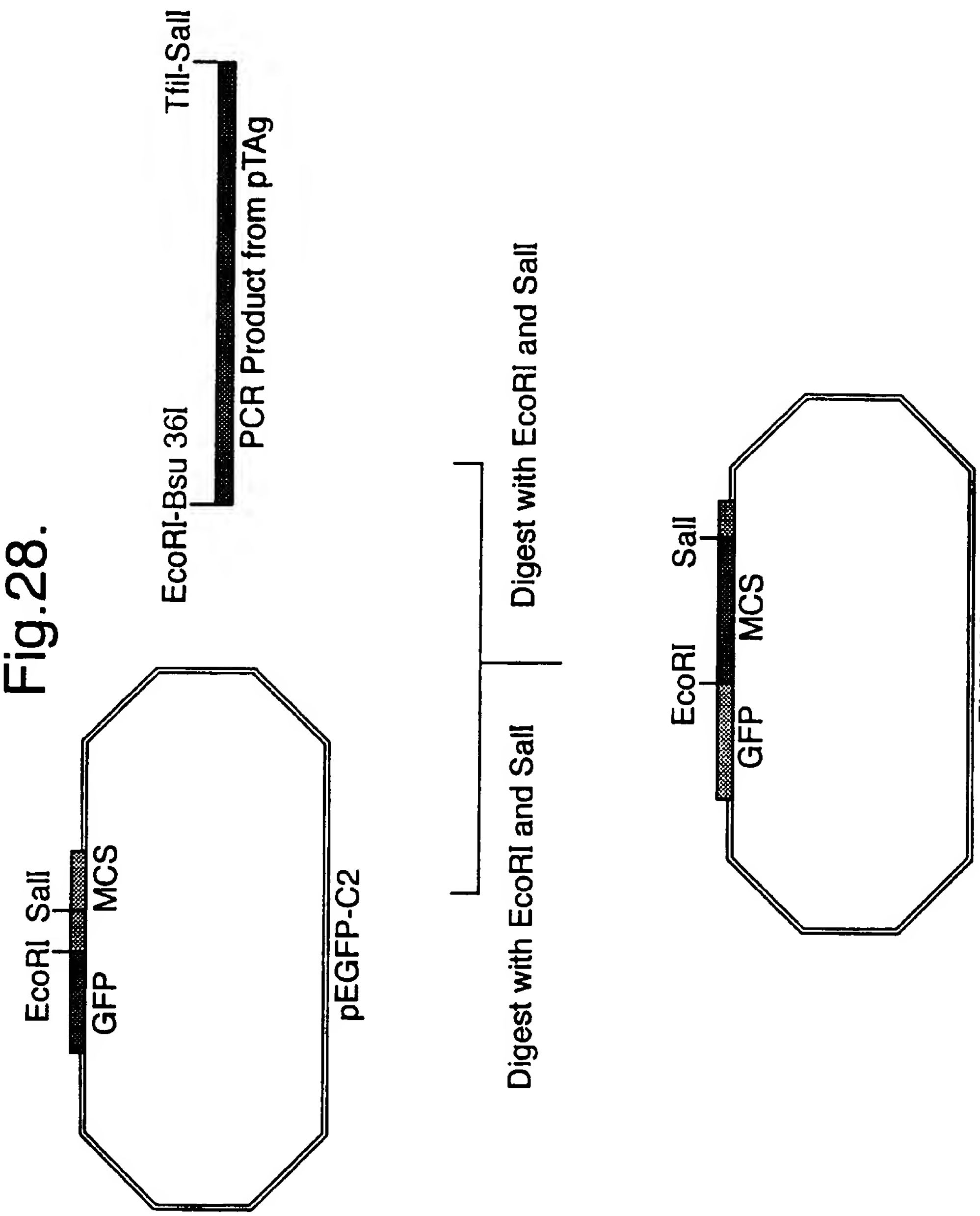

BglII
SalI
GFP
MCS
pEGFP-C2
Digest with BglII and SalI
BamHI
XbaI-SalI
Bsu 36I
pEVV187
Digest with BamHI and SalI
SalI
BglII
Bsu 36I
GFP
pEVV186

Fig.27.

Fig.28.


TfiI-SalI
PCR Product from pTAg
EcoRI-Bsu 36I
EcoRI
SalI
GFP
MCS
pEGFP-C2
Digest with EcoRI and SalI
Digest with EcoRI and SalI
SalI
EcoRI
MCS
GFP
pEVV172

INTERNATIONAL SEARCH REPORT

Intern   ıal Application No
PCT/GB 99/00874

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/18 C12N15/33 A61K39/193

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 98 53077 A (WALTER REED ARMY INST RES) 26 November 1998 (1998-11-26) abstract page 11, line 1 - page 12, line 19 page 19, line 33 - page 20, line 14 ---	1-15
Y	US 5 185 440 A (JOHNSTON ROBERT E ET AL) 9 February 1993 (1993-02-09)	1,3-5
A	the whole document ---	10-15
Y	JAKOB R.: "Nucleolar accumulation of core protein in cells naturally infected with Semliki Forest virus" VIRUS RESEARCH, vol. 30, no. 2, 1993, pages 145-160, XP002110934 cited in the application the whole document ---	1,3-5

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 August 1999	09/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KINNEY R.M. ET AL.: "The full-length nucleotide sequences of the virulent trinidad donkey strain of venezuelan equine encephalitis virus and its attenuated vaccine derivative strain TC-83" VIROLOGY, vol. 170, no. 1, 1989, pages 19-30, XP002110935 ORLANDO US cited in the application the whole document ---	1-15
A	KUHN R. ET AL.: "Attenuation of Sindbis virus neurovirulence by using defined mutations in nontranslated regions of the genome RNA" JOURNAL OF VIROLOGY, vol. 66, no. 12, 1992, pages 7121-7127, XP002110936 the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/GB 99/00874

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9853077 A	26-11-1998	AU 7501898 A	11-12-1998
US 5185440 A	09-02-1993	NONE	